Visual memory in *Drosophila melanogaster*

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ABSTRACT

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Despite their small brains, insects are capable of incredible navigational feats. Even *Drosophila melanogaster* (the common fruit fly) uses visual cues to remember locations in the environment. Investigating sophisticated navigation behaviors, like visual place learning, in a genetic model organism enables targeted studies of the neural circuits that give rise to these behaviors. Recent work has shown that the ellipsoid body, a midline structure deep within the fly brain, is critical for certain navigation behaviors. However, nearly all aspects of visual place learning remain mysterious. What visual features are used to encode place? What is the site of learning? How do the learned actions integrate with the core navigation circuits?

To begin to address these questions I have established an experimental platform where I can measure neural activity using a genetically encoded calcium indicator in head-fixed behaving *Drosophila.* I further developed a virtual reality paradigm where flies are conditioned to prefer certain orientations within a virtual environment. In dendrites of ellipsoid body neurons, I observe a range of specific visual responses that are modified by this training. Remarkably, I find that distinct calcium responses are observed during presentation of preferred visual features. These studies reveal learning-associated neural activity changes in the inputs to a navigation center of the insect brain.

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Finally, I want to thank members of my thesis committee, past and present, for their feedback, direction, and time: Larry Abbott, Rudy Behnia, Carlos Brody, Gaby Maimon, Nate Sawtelle, and,Gary Struhl.

Dedication

This dissertation is dedicated to my parents, to my brother Peter, and to Abbie.

PREFACE

This thesis describes our effort to understand how visual memories are stored in the fly brain. It is organized into five chapters. Chapter 1 presents the development of optogenetic activation of thermoreceptors to replace heat for use in head-fixed imaging experiments. This technique is critical for the experiments of the subsequent chapters. Chapter 2 described the development of a novel head-fixed visual learning assay that is compatible with calcium imaging, and establishes the necessity of a population of ellipsoid body neurons for visual learning. Chapter 3 investigates the response properties of these neurons. Chapter 4 examines the activity changes of these neurons, within the brain of a single animal, before and after learning in the head-fixed assay. Chapter 5 summarizes and discusses the work.

Chapter 1
Virtual Heat

1.1 Background

Despite their compact brain, flies are capable of impressive behavior. Recent work in the lab demonstrated the ability of flies to use visual cues to remember a cool, safe location within a hot, aversive thermal environment (Ofstad, *et al.,* 2011). A targeted genetic silencing screen revealed neurons within the ellipsoid body, a structure deep within the fly brain, to be required for this task. Still, nearly all aspects of visual learning in flies remains a mystery. What is the functional role of ellipsoid body neurons during learning and recall? Are their responses modified by learning? How are visual memories acquired or stored in the fly brain?

To answer these questions, we wanted to port the free-walking visual-thermal learning assay to an experimental platform compatible with 2-photon calcium imaging. Our platform had to provide both visual and thermal stimuli. Providing visual stimuli to a head-dissected fly, and imaging calcium responses, is now a mature method within the fly vision field (Reiser & Dickinson, 2008; Strother *et al.,* 2014).

Established methods for delivering thermal stimuli to an insect imaging preparation are relatively less common. Though a few examples exist in the literature (Gallio *et al*, 2011; Frank *et al.,* 2015), we observed several problems attempting to heat a fly and image GCaMP responses in central brain neurons. These problems include: outgassing of the fly saline, apparent z-motion of the sample, direct temperature-related effects on GCaMP fluorescence, and most importantly, poor preparation survivability. Outgassing, or bubbles forming in the saline between the sample and the objective, results from gas solubility decreasing in heated saline, and leads to poor image quality. Apparent z-motion of the sample could result from a number of factors, including heating of the objective components (changing the focal length of the objective); heating the saline and altering the refractive index of the saline; and thermal expansion of the preparation

holder. Temperature-dependent fluorescence effects likely result from the inherent temperature dependence of calmodulin calcium affinity (Gangola & Pant, 1983); recall that GCaMP is a protein construct of circularly-permuted GFP and calmodulin. Strategies exist to deal with some of these problems; however, poor preparation survival is limiting. We sought a cleaner strategy to deliver heat stimuli.

The biological need to stay in a thermal comfort zone is a powerful driver of behavior. Temperature is a particularly salient stimulus for *Drosophila*, who (like other arthropods) do not internally regulate body temperature. Instead, they seek out preferred temperature zones in their environment. The preferred temperature range of fruit flies is narrow (24°C +/- 2 degrees) and thermotaxis is easy to elicit and measure in the lab; therefore, the sensory neurons and thermosensitive proteins underpinning *Drosophila* thermal preference have been the subject of several interesting studies. Such work has led to a well-developed understanding of the inputs to the *Drosophila* thermosensory system. To sense heat, adult *Drosophila* use three known neural cell types, with unique thermosensory proteins tuned to unique temperature ranges and heating rates. These include the AC neurons, located in the central brain, which express dTRPA1 and respond to slow increases in temperature from 28-32°C (Hamada *et al*, 2008). These neurons are particularly interesting, because they are both central brain interneurons and primary sensory neurons. Neurons expressing the *painless* receptor are distributed throughout the body, and mediate rapid avoidance from high heat environments, above 37°C (Tracey, *et al*., 2003).

Perhaps most relevant for the visual-thermal place learning task are the so-called “Hot-Cells” (Figure 1.1) which reside in the arista, or the fine comb-like structure which projects off the most distal segment of the antenna. Hot Cells express the receptor Gr28.b and respond to

rapid temperature increases at or around 35°C – the aversive temperature used in the free-walking place learning assay (Gallio *et al*., 2011, Ni *et al,* 2013, Ofstad *et al.,* 2011). Due to the problems we observed with "real" heat in imaging preparations, we wanted to test if optogenetic activation of Hot Cells could replace heat for thermal avoidance and ultimately learning.

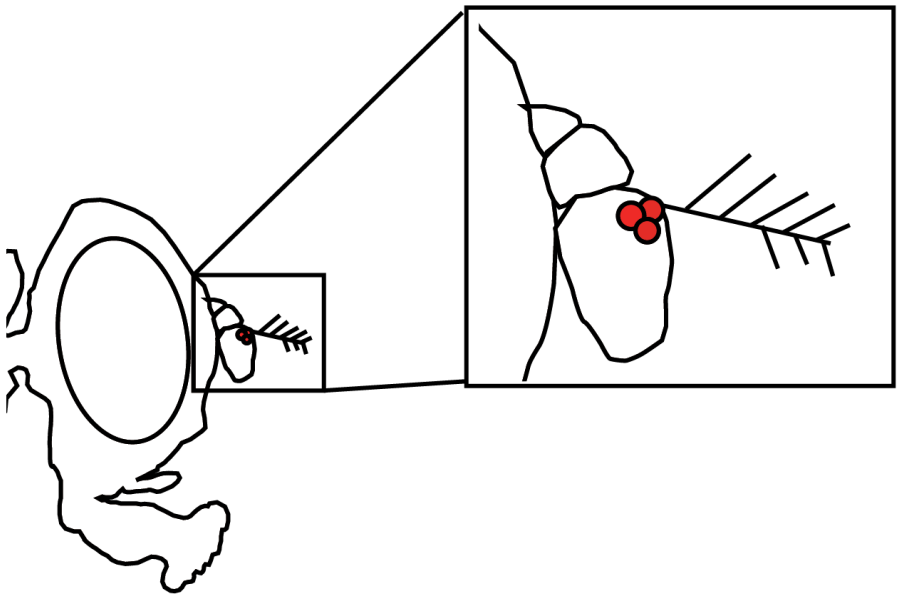


Figure 1.1: Drosophila Hot Cells. Hot cells reside in the third segment of the fly antenna. They are responsible for sensing heat, at around 35C. (Adapted from Ni *et al*., 2013)

1.2 Results

To test if optogenetic Hot Cell activation could drive avoidance behavior, we designed a two-by-two avoidance experiment, similar to those used to measure "real" thermal preference in flies. In our assay, however, red light replaces heat. Briefly, single flies expressing CsChrimson (Klapoetke, *et al.*, 2014) in Hot Cells were tracked in real-time as they walked across a circular platform. When flies entered two of four opposite quadrants, we expose the fly to red (625 nm) light. We observe fly behavior to determine if flies avoid light exposure. After one minute, we swap quadrants, and over the course of an experiment, we sweep through a range of light intensities. As demonstrated in Figure 1.2, we observe an intensity-dependent light avoidance that saturates at 12 uW/mm^2. Control flies that do not express CsChrimson in Hot Cells exhibit no such avoidance. The magnitude of light avoidance is similar to what has been reported for flies at 35°C, consistent with the relevant biological range reported for these neurons. Hot Cell activation, like "real" heat, drives avoidance in fruit flies.

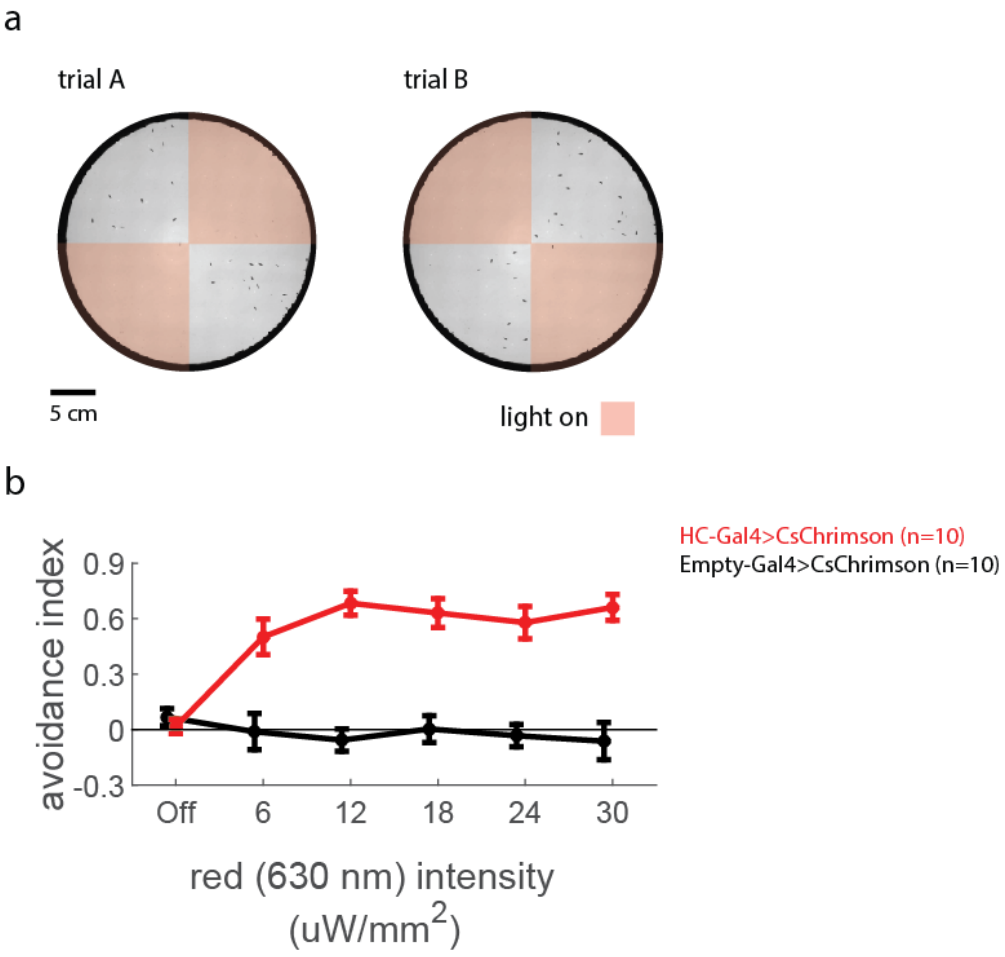


Figure 1.2: Optogenetic activation of Hot Cells drives avoidance behavior. Flies expressing CsChrimson in Hot Cells were exposed to red (625 nm) light in two of four quadrants in a freewalking 2x2 avoidance assay. After 1 minute, these quadrants were flipped. **(a)** Shown is a composite image of twelve trials for three flies. Flies clearly avoid red light exposure. **(b)** Light avoidance is intensity-dependent and saturates at 12 uW/mm^2.

Hot-cell activation is sufficient for avoidance, but is it sufficient for learning? To test this, we built an optogenetic version of the free-walking visual place learning assay. We define a "safe zone" within one quadrant of the arena platform. Outside the safe zone, flies are exposed to red light. We surround the platform with a circular LED panorama, on which we display static visual cues. At the beginning of each training trial, the location of the safe zone and the pattern

position on the panorama rotate +/- 90 degrees. The absolute position of the safe zone changes, but the relative location of the safe zone to the visual cues remains constant. We challenge the flies to use the visual cues to learn the location of the safe zone. After ten training trials of one minute each, we test flies with a "probe" trial. A probe trial begins identical to training trials, with one critical difference: no new safe zone appears. The arena is uniformly lit by red light. We observe fly behavior to see if flies search for the missing safe zone at the appropriate location.

As seen in Figure 1.3, flies do develop a place preference. During the probe trial, flies search for the missing safe zone at the entrained location. Flies trained in a control protocol, during which pattern rotation is "uncoupled," or decorrelated from the safe zone, exhibit no such search bias. Excitingly, optogenetic Hot Cell activation can replace heat for *Drosophila* place learning. This "virtual heat" provides a cleaner strategy for delivering thermal stimuli to a fly during an imaging preparation.

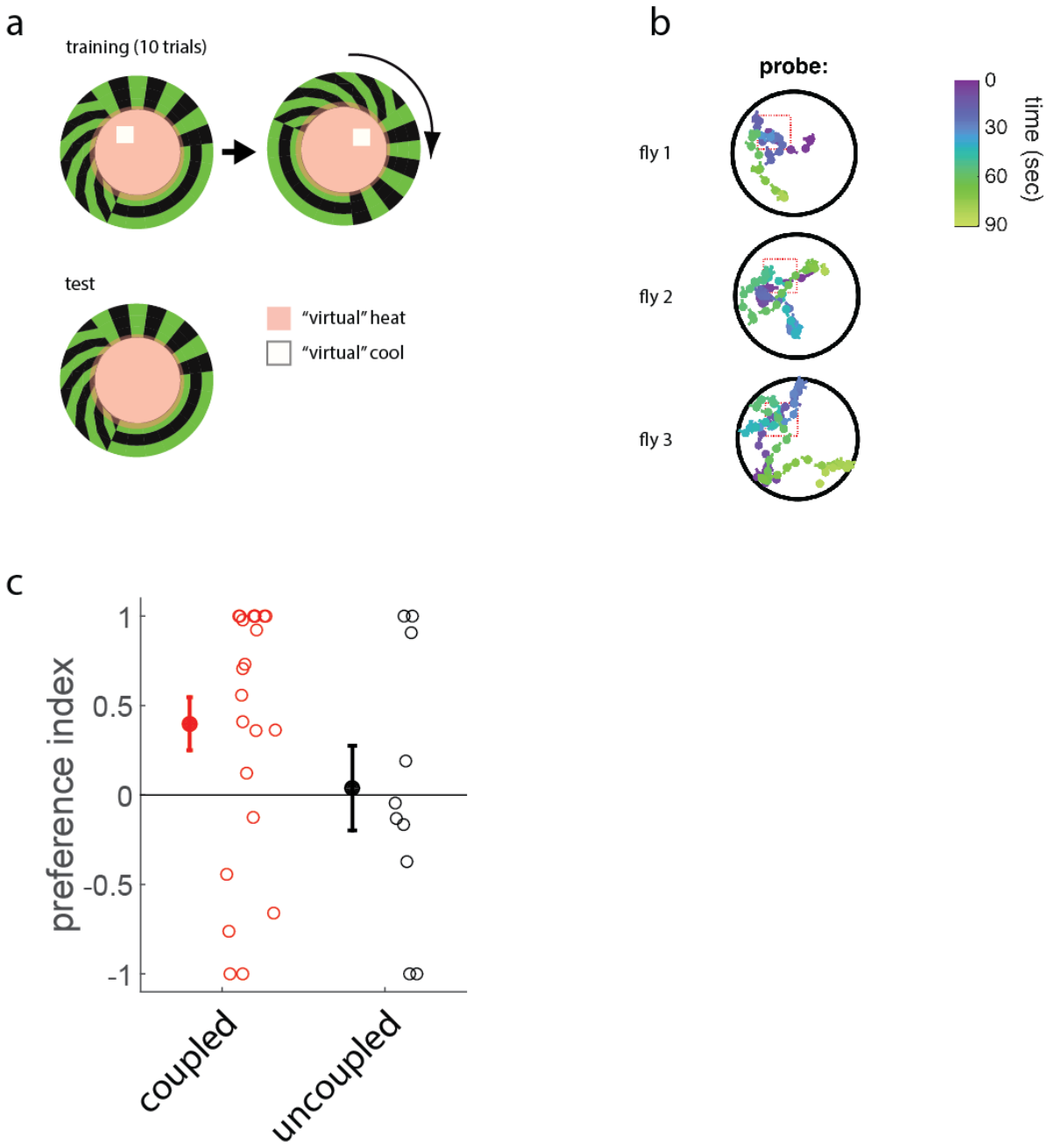


Figure 1.3: Optogenetic place learning. Optogenetic activation of Hot Cells is sufficient to drive visual place learning in *Drosophila*. **(a)** Shown is an overview of the experimental protocol. We use red light (625 nm) to simulate a virtual thermal landscape for a free-walking fly. We create a light-free (virtual cool) region within one quadrant, and challenges flies to learn to use visual cues to locate it. After training, we test flies in a uniformly red-lit environment, and observe walking trajectories to see if they search for the missing cool zone at the appropriate location. **(b)** Shown are example probe trajectories for three flies in the optogenetic place learning assay. Flies had been trained to locate the cool zone within the dashed red box. **(c)** Shown is summary data for the experiment, Trained flies exhibit a profound search bias at the former location of the cool zone; flies exposed to a protocol without consistent visual cues (uncoupled) do not. (coupled, n=20 flies; uncoupled n=10 flies)

1.3 Methods

Fly stocks

Adult female flies were used 3-5 days post ecclosion. Full genotypes are as follows:

HC>CsChrimson: *w; HC-Gal4/+; UAS-CsChrimson-mCherry (su(Hw)attP1)/+*

Empty-Gal4>CsChrimson: *w; +/+; UAS-CsChrimson-mCherry (su(Hw)attP1)/ pDPGal4U(attP2)*

Experimental Platform

The base of the experimental platform is a lightbox (SmartVision MOBL-300x300 625/825) which provides near-IR (825 nm) light for fly tracking, and red (625 nm) light for activating CsChrimson. Flies were confined to the platform with a piece of glass, coated with silicone solution to discourage flies from clinging to the surface. A custom-machined ring encircled the arena, and was heated to keep flies off the edge. For place learning experiments, a modular circular LED display (described citation) was placed directly onto the glass.

Tracking

Single flies were recorded in real time using a digital camera (Point Grey FL3-U3-13Y3M-C) with near-infrared longpass filter (Hoya R72). Fly positions were tracked using Kalman-filtered background subtraction at 50 hz. Tracked positions were passed to a light-intensity lookup table. If the fly resided in a punished position, the fly was flashed with red light for the duration of that frame.

Behavioral Metrics

Avoidance assay: for each trial, we calculate an "avoidance index" to quantify avoidance behavior.

Avoidance index = $(\text{time}^{\text{Light On}} - \text{time}^{\text{Light Off}})$/total time. Avoidance index can range from 1 (perfect avoidance of light) to -1 (entire trial spent in lit quadrant) .

Place learning assay: for each trial, we compute several behavioral metrics, including time to safe zone, time at safe zone, etc. For the probe trial, we quantify search behavior using a "quadrant preference index," similar to the avoidance index.

Preference index =

$$(\text{time}^{\text{target quadrant}} - \text{time}^{\text{opposite quadrant}})/ (\text{time}^{\text{target quadrant}} + \text{time}^{\text{opposite quadrant}}).$$

Where applicable, tests for statistical significance were performed using a Student's T-test. All analysis was performed with MATLAB.

Chapter 2

Visual learning
in virtual reality

2.1 Background

To investigate how visual learning is implemented in the fly brain, we wanted to measure neural activity before, during, and after a visual learning task. This required developing a head-fixed, fly-on-the-ball preparation compatible with two-photon calcium imaging.

Here we draw on the long history of tethered fly behavior, as well as recent developments in head-fixed imaging. For decades, neuroscientists have been gluing insects to sticks and observing their behavior as they fly in place or walk on balls (Buchner, 1976). These tethered preparations have led to insights in motion vision (Poggio & Reichardt, 1976), figure discrimination (Liu *et al.,* 2006), and chemotaxis (Duistermars & Frye, 2008). In 1996, the Heisenberg published an assay demonstrating that a tethered, flying fly can discriminate visual patterns, and learn to associate patterns with punishment (Wustmann, *et al*., 1996). This assay was then used to identify brain structures necessary for visual learning in the fly (Liu *et al.,* 2006). Everything about the function of these circuits remained a mystery.

A recent trend in *Drosophila* neuroscience is combining tethered walking and flying assays with head-open preparations for electrophysiology and multiphoton calcium imaging. Such a platform enables the study of neural activity during naturalistic behavior. These experiments have resulted in several exciting observations. For example, the lobula plate tangential cells (well-studied motion-sensitive optic lobe interneurons) exhibit large gain changes in a behaving versus a quiescent fly (Chiappe *et al.*, 2010 Maimon *et al*, 2010). These same neurons appear to carry efference-copy signals, counter-acting self-generated motion from volitional movements (Kim, *et al.,* 2017). Elsewhere in the fly brain, neurons within the ellipsoid body of the central complex provide a read-out of the angular heading of the fly (Seeling & Jayaraman, 2016). To date, these experiments utilize robust, but relatively simple, fly behaviors

– flying vs. not flying, optomotor turning, or stripe fixation. When we began our experiments, it was an open question whether more nuanced behaviors, like tethered visual learning, could be compatible with a head-open physiology preparation.

To study activity changes associated with visual memory in the fly, we developed a head-fixed tethered analog of the free-walking place learning experiment. Although we wanted to replicate, as much as possible, the original assay, we also wanted to simplify and constrain the task to facilitate interpretability with calcium signals. The resulting experiment draws inspiration from both the visual place learning and Heisenberg tethered-flight learning assays. To avoid the problems observed using "real" heat with imaging, we use optogenetic activation of antennal Hot Cells as an instructive stimuli.

Briefly, flies expressing CsChrimson in Hot Cells and GCaMP6f in ellipsoid body neurons are cold-anesthetized and glued to a physiology shim filled with insect saline. We open a hole in the head, and position the fly atop an air-supported foam ball. As the fly walks, the ball rotates; ball rotation is tracked to record the intended turning behavior of the fly. This assembly sits at the center of a 180° circular LED display; ball rotation updates visual features on the screen as if the fly were moving through the natural world. An optical fiber is placed directly in front of the fly to deliver 660 nm light, for delivering "virtual heat" by activating CsChrimson in Hot Cells (Figure 2.1a).

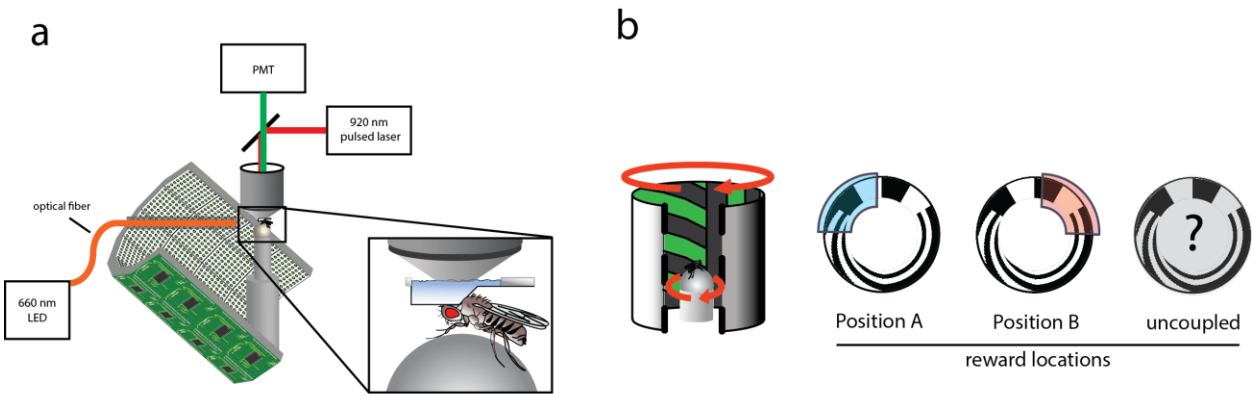


Figure 2.1: Imaging-compatible head-fixed visual learning. (a) A diagram of the behavioral platform. Flies are anesthetized, head-fixed, and dissected for imaging before being placed on the ball. A 180° x 120° circular LED display provides visual cues; an optical fiber delivers red (660 nm) light for Hot Cell activation. Flies are imaged from above. **(b)** We use this platform to simulate a patterned cylinder for the fly. The fly controls its orientation within the cylinder by rotating the ball. We train two sets of flies to different positions along the cylinder pattern. Position A flies are trained to seam between diagonal and vertical stripes. Position B flies are trained to the seam between vertical stripes and horizontal bars. In the uncoupled control condition, the virtual cool zone appears randomly within the cylinder during training trials.

We simulate a virtual patterned cylinder for the fly. By turning on the ball, the fly controls its orientation within the cylinder (Figure 2.1b). With virtual heat, we define a thermal environment within the cylinder: the fly can only escape virtual heat if it orients within one of four quadrants. We challenge flies to learn the visual cues associated with this "cool zone" over the course of 15 one-minute training trials. To measure within-fly changes in visual preference, we present flies with a "probe trial" before and after training. During probe trials, the thermal environment within the cylinder consists of uniform virtual heat. If flies use visual cues to identify the cool quadrant, they should develop a preference for visual features of that quadrant.

2.3 Results

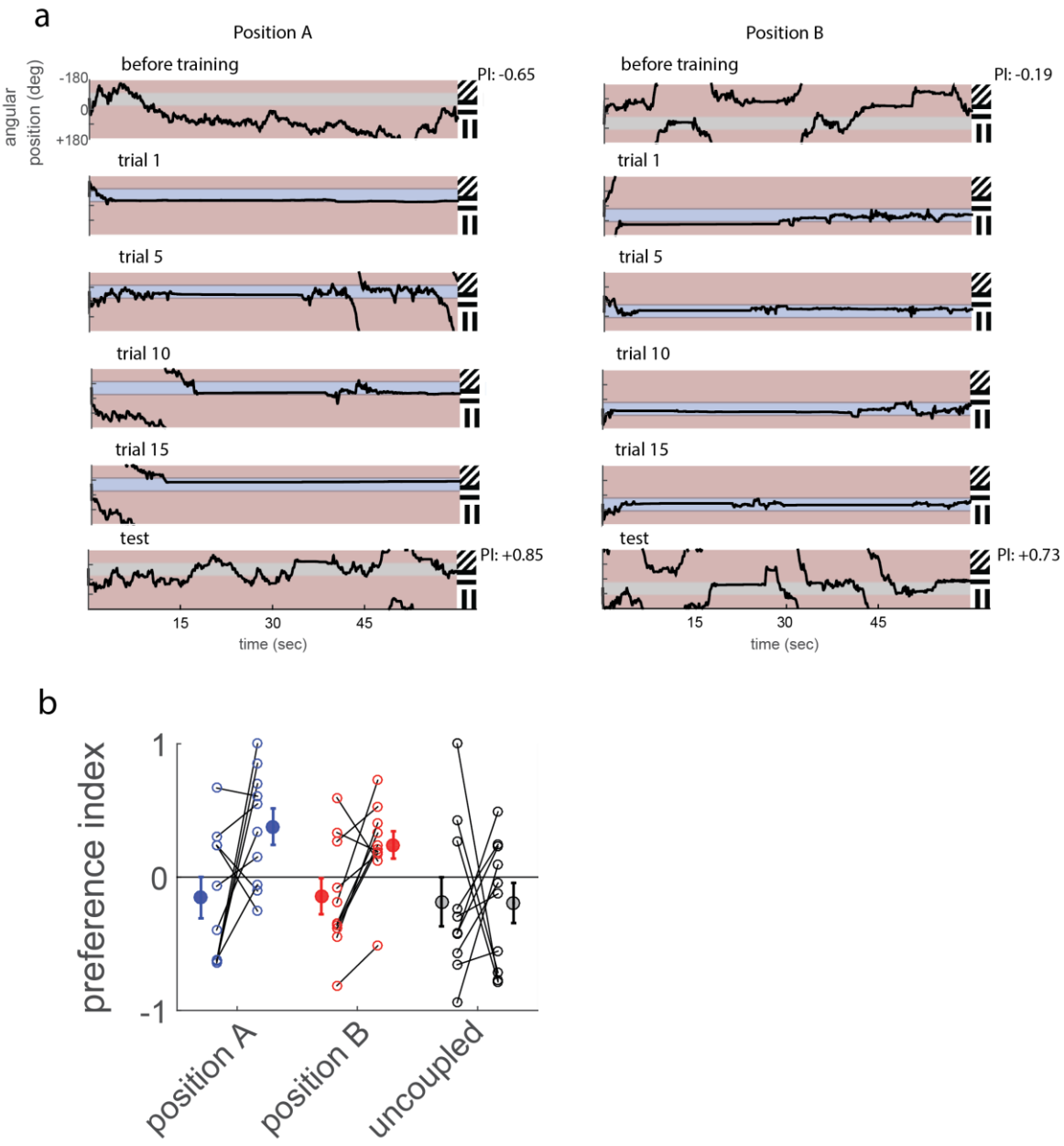


Figure 2.2: Head-dissected flies learn the visual association task. (a) Examples of single-fly behavior in the visual learning assay. Fly orientations are plotted through time. During training trials, flies rapidly reach the virtual cool zone and largely remain within it. From before-training to test trials, flies acquire a dramatic preference for the previously rewarded visual cues, even in the absence of the virtual cool zone. **(b)** Learning summary data. Increased preference for rewarded visual cues, from pre-training to test, is observed across flies. This is not observed in uncoupled controls

As demonstrated in figure 2.2, naïve flies exhibit no consistent pattern preference before training. During training, flies rapidly locate the cool zone and remain within it for most of the trial. We do not observe improvement in metrics for reaching the cool zone during training. This could be due to the reduced complexity of the tethered behavior – even undirected flies can reach the cool zone relatively quickly. We do observe a dramatic preference for the rewarded quadrant following training. Flies trained under an uncoupled control condition, in which the cylinder pattern is randomly shifted during training, develop no such preference.

In the original free-walking assay, a targeted silencing screen identified the ellipsoid body (EB) of the central complex as being required for visual place learning. Is the ellipsoid body also required for tethered visual learning in *Drosophila*? Because we are using the Gal-4 system to drive CsChrimson expression, we require a non-overlapping expression system to drive expression of other transgenes in neurons of interest. We identified candidate lexA lines from the Rubin collection (Jenett, *et al.*, 2012) with expression in the ellipsoid body. One of these, 11F03, demonstrated partial expression overlap with lines known to be required for the original free-walking assay. To test if these EB neurons are required for the tethered assay, we used the 11F03 line to drive expression of Shibire^{TS}. Shibire^{TS}, a temperature-sensitive dynamin mutant, interferes with synaptic transmission at temperatures above 28°C (Dubnau, *et al.*, 2001). Silencing 11F03 neurons abolishes learned orientation preference (Fig 2.3). As in the free-walking assay, neurons within the ellipsoid body are required for tethered visual learning. We focused future imaging studies on these 11F03 neurons.

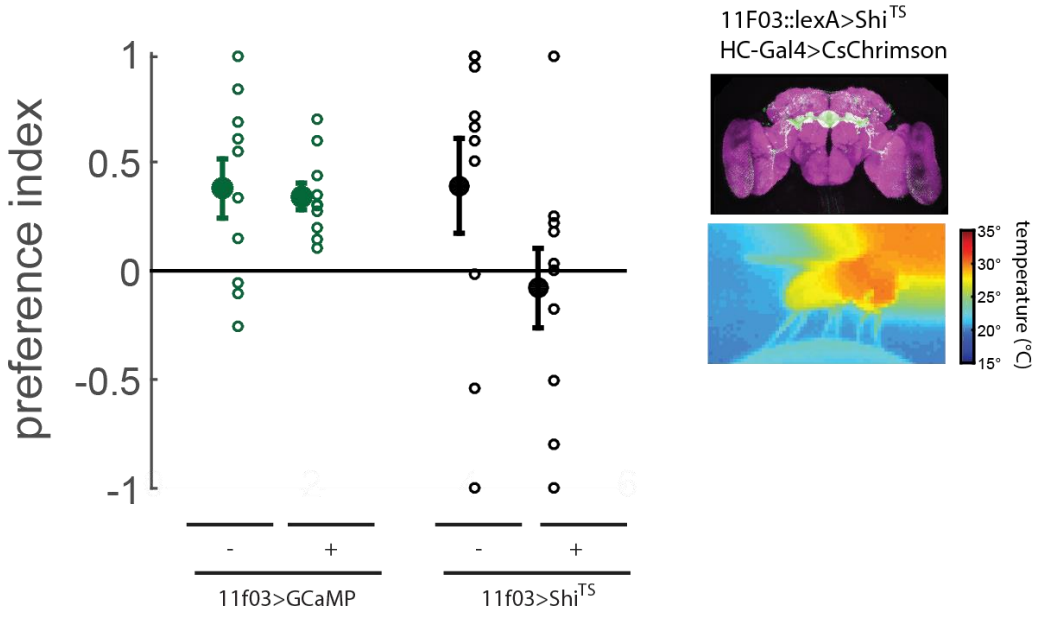


Figure 2.3: 11F03 ellipsoid body neurons are required for tethered visual memory. (a) Silencing synaptic output of 11F03 neurons with temperature-sensitive Shibire abolishes visual memory in warmed flies (+), but not in room-temperature flies (-), or flies expressing GCaMP. Shibire-mediated silencing only occurs at elevated (restricted) temperatures. **Top right:** Expression pattern of 11F03-lexA **Bottom right:** Thermal image of heated fly.

2.3 Methods

Fly stocks

Adult female flies were used 3-5 days post ecclosion. Full genotypes are as follows:

HC>Chr, 11F03>GCaMP6f: *w^+(DL)/w; 11F03-LexAp65(attP40)/HC-Gal4; UAS-CsChrimson-mCherry (su(Hw)attP1), pGP-JFRC59-13XLexAop2-IVS-p10-GCaMP6f (VK00005)/+*

HC>Chr, 11F03>Shi^{TS}:

w^+(DL)/w; 11F03-LexAp65(attP40)/HC-Gal4; UAS-CsChrimson-mCherry (su(Hw)attP1)/ pJFRC104-13XLexAop2-IVS-Syn21-Shibire^{ts1}-p10 (su(Hw)attP1)

Experimental Platform

The experimental platform consists of the Janelia Fly Treadmill System (flyfizz.org, Seelig *et al.*, 2013), combined with 180° insect vision LED display (Reier & Dickinson, 2008). The display is angled at 60° to compensate for the head tilt of the physiology preparation. An optical fiber delivers light to the fly from a Thorlabs 660nm LED (Thorlabs M660F1). Flies are imaged with a Thorlabs Bergamo II 2-photon imaging system with a SpectraPhysics Mai-Tai DeepSee excitation laser source at 920 nm from 5-10 mW.

Behavior Assay

We simulate a cylindrical environment for the fly, with visual patterns (vertical, horizontal, and diagonal stripes) within the walls of the cylinder. Through optogenetic activation of Hot Cells, we create an angular thermal environment for the fly. The fly controls its orientation within the cylinder through closed-loop rotation of the pattern and thermal environment, coupled to rotation of the ball. The simulation is implemented in MATLAB and runs at 50hz.

An experiment consists of a baseline trial, 15 training trials, followed by a test trial. All trials are one minute each. Baseline and test trials are conducted in a uniform "virtual heat" environment. In training trials, one quadrant contains a virtual cool zone. Flies begin each trial at +/- 90 ° from the cool zone. We trained two sets of flies to different locations within the cylinder. "Position A" flies were trained to the seam between diagonal and vertical stripes. "Position B" flies were trained to the seam between horizontal and vertical stripes. We trained a separate set of flies to an uncoupled control condition, in which the cylinder pattern is shifted randomly relative to the cool zone. These flies do not receive a consistent cue associated with the cool

quadrant.

Behavior Analysis

Orientation behavior was analyzed with custom scripts in MATLAB. We define a quadrant preference index as:

Preference index =

$$(\text{time}^{\text{target quadrant}} - \text{time}^{\text{opposite quadrant}}) / (\text{time}^{\text{target quadrant}} + \text{time}^{\text{opposite quadrant}}).$$

Silencing experiments

Silencing experiments were conducted with un-dissected flies head-fixed to the physiology shim. To heat the fly head, warm water was flowed through the saline reservoir using a liquid temperature controller (Warner Instruments CL-100). Heating was calibrated with thermal imaging. Flies expressing either GCaMP6 or temperature-sensitive Shibire were trained at 20 degrees (permissive temperature, no silencing) or 28°C (restrictive temperature for silencing).

Chapter 3

Activity in ellipsoid body ring neurons

3.1 Background

Neurons labeled by the 11F03-lexA line are required for visual memory in *Drosophila.* What do these neurons do? We investigated the response properties of 11F03 neurons in both traditional stimulus-response experiments and during closed-loop visual learning behavior.

The 11F03 line drives strong expression, broad expression in ring neurons of the *Drosophila* ellipsoid body. The ellipsoid body is a strikingly ring-like structure within the central complex of the fly brain. The central complex itself is a network of densely recurrent midline neuropil deep within the brain, and traditionally includes the protocerebral bridge, fan-shaped body, and noduli in addition to the ellipsoid body (EB) (Hanesch, *et al*., 1989). Ring neurons are so-called because they innervate concentric, circular rings within the EB. Ring neurons receive input at glomerular dendrites within a structure lateral to the EB called the "bulb."

Little is known about the function of *Drosophila* central complex neurons. Until recently, the best-characterized examples of insect central complex neurons came from electro-physiological studies in other species. For example, neurons tuned to the e-vector of polarized light have been found in the central complex of the locust (Heinze & Homberg, 2007). Within the central complex of the Monarch butterfly *Danaus plexippus*, neurons are strongly tuned to the azimuthal position of bright, sun-like objects (Heinze & Reppert, 2011). In both species, these cell types are thought to play a role in long-distance navigation by providing the animal with a "sky compass" tuned to global features of the daytime sky.

In recent years, the development of the *Drosophila* behavior-compatible calcium imaging preparation, and improvements in genetically-encoded calcium indicators, have catalyzed the study of central complex neurophysiology in the fruit fly. One study, which investigated the

activity of central complex neurons across the brain region in flying flies, found differential responses to broad visual stimuli and evidence for behavioral-state modulation (Weir & Dickinson, 2015). A different study of columnar "wedge" neurons within the EB (and posterior to ring neurons) found dramatic evidence that the EB tracks the angular heading of the fly, in an analogous manner to head direction cells in the mammalian entorhinal cortex (Seelig & Jayaraman, 2016; Taube, *et al*., 1990).

Most relevant for our neurons of interest, a study from the Jayaraman lab investigated the visual response properties of EB ring neurons, at their inputs within the bulb (Seelig & Jayaraman, 2013). They discovered these neurons have restricted receptive fields that map visual space. Each receptive field has excitatory and inhibitory subfields, which confer selective orientation tuning. Therefore, these neurons are proposed to be fly brain feature detectors.

Recall that the bulb contains dendrites of EB ring neurons. Hartenstein and colleagues (Omoto, *et al.,* 2017) demonstrated that the bulb contains anatomical sub-structures, called the superior, anterior, and inferior bulbs. These sub-fields are developmentally and functionally distinct, and receive inputs from adjacent but non-overlapping regions of the anterior optic tubercle (AOTu). These regions in turn send projections to separate sub-rings within the ellipsoid body.

This body of work on central complex neurons demonstrates that these cells have properties important for supporting complex behaviors, like vision-guided navigation. Our current neurons of interest, labeled by the 11F03 line, are also EB ring neurons. At present we are unsure of the relationship between 11F03 neurons and those previously studied by the Jayaraman group – they could be a subset, or a distinct population entirely. (We are currently in the process of exploring this with double-labeling experiments.) To develop and understanding

of the functional role of these neurons, we set out to study their response properties.

3.2 Results

We used two-photon imaging to record calcium responses in 11F03 neurons expressing GCaMP6m. We focused our study on dendrites within the within the bulb, as no clear calcium responses were ever observed in the ellipsoid body ring. This may be due to the fact that axon terminals within the ring are fine and intermingled. Within the bulb, the glomerular organization of the dendrites provides convenient, defined regions of imaging for analysis.

We first presented a battery of visual stimuli to a passive, immobile fly. Clear visual responses were observed in dendrites within the superior compartment of the bulb. These responses are collected in Figure 3.2[1]. 11F03 neurons respond to visual stimuli in the ipsilateral visual hemisphere. They are on-selective, meaning they respond strongly to bright objects, and weakly (or not at all) to dark ones. In response to looming (circular disk expansion), we observe responses classically associated with center-surround inhibition: for bright disks, there is early excitation to small disks, followed by response drop-off as the disk grows. For dark disks, we observe early inhibition to dark disks followed by late excitation. These neurons display no strong directional selectivity to moving bars. They do exhibit speed tuning, and respond more strongly to slow visual stimuli. Overall, the strongest responses observed were to small, bright objects swept across the visual field at 30 degrees per second.

[1] The quality of these recordings is poor. These were some of our first imaging experiments.

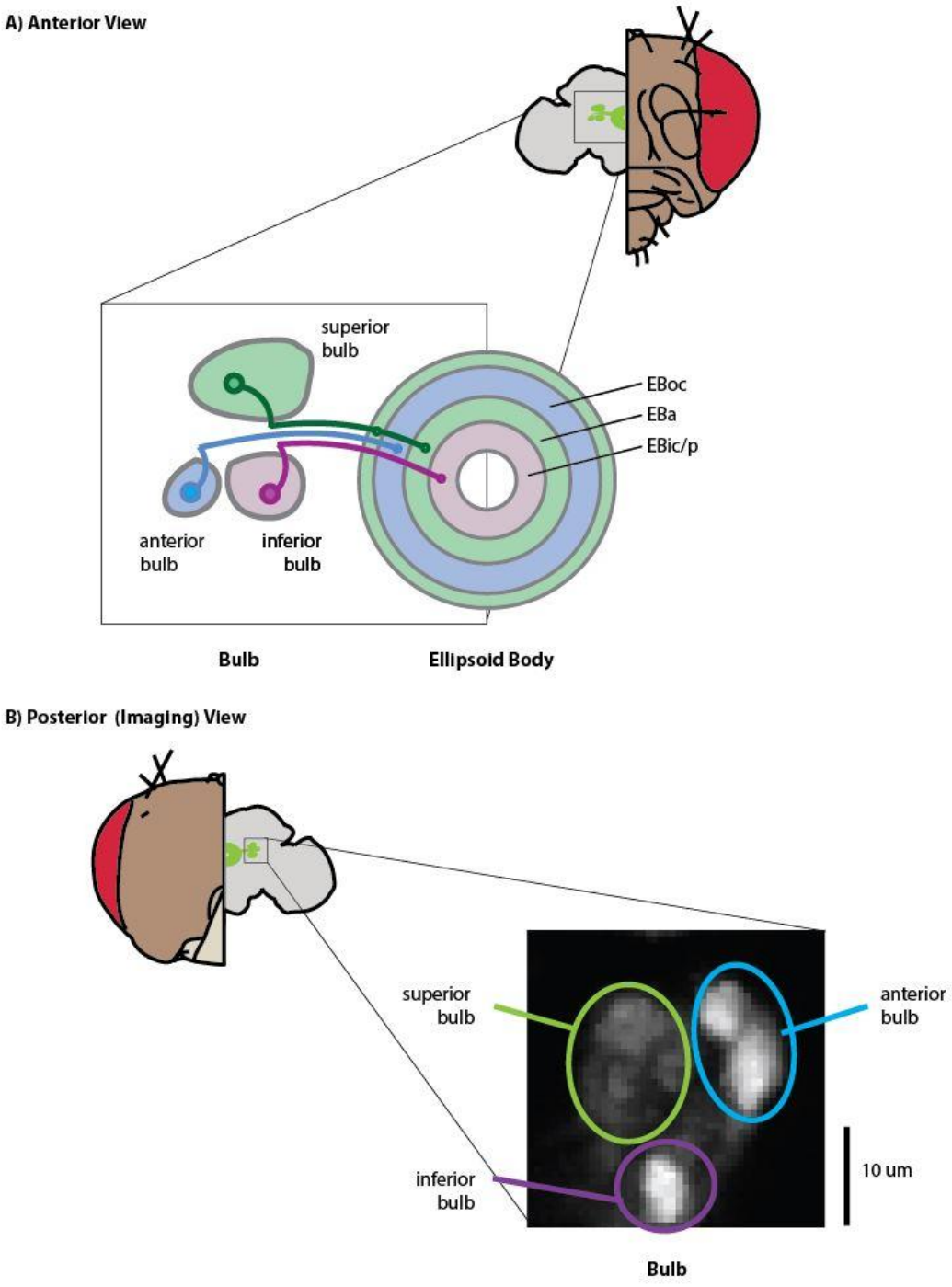


Figure 3.1: Anatomy of ellipsoid body ring neurons. **(a)** Ellipsoid body ring neurons have dendrites in a lateral neuropil called the bulb. The bulb is composed of superior, anterior, and inferior subfields, which project to distinct rings within the ellipsoid body. EBoc = outer central, EBa = anterior, EBic/p = interior central/posterior (adapted from Omoto, *et al*., 2017). **(b)** We image the brain posteriorly; 11F03 drives expression of gCaMP in all three subfields.

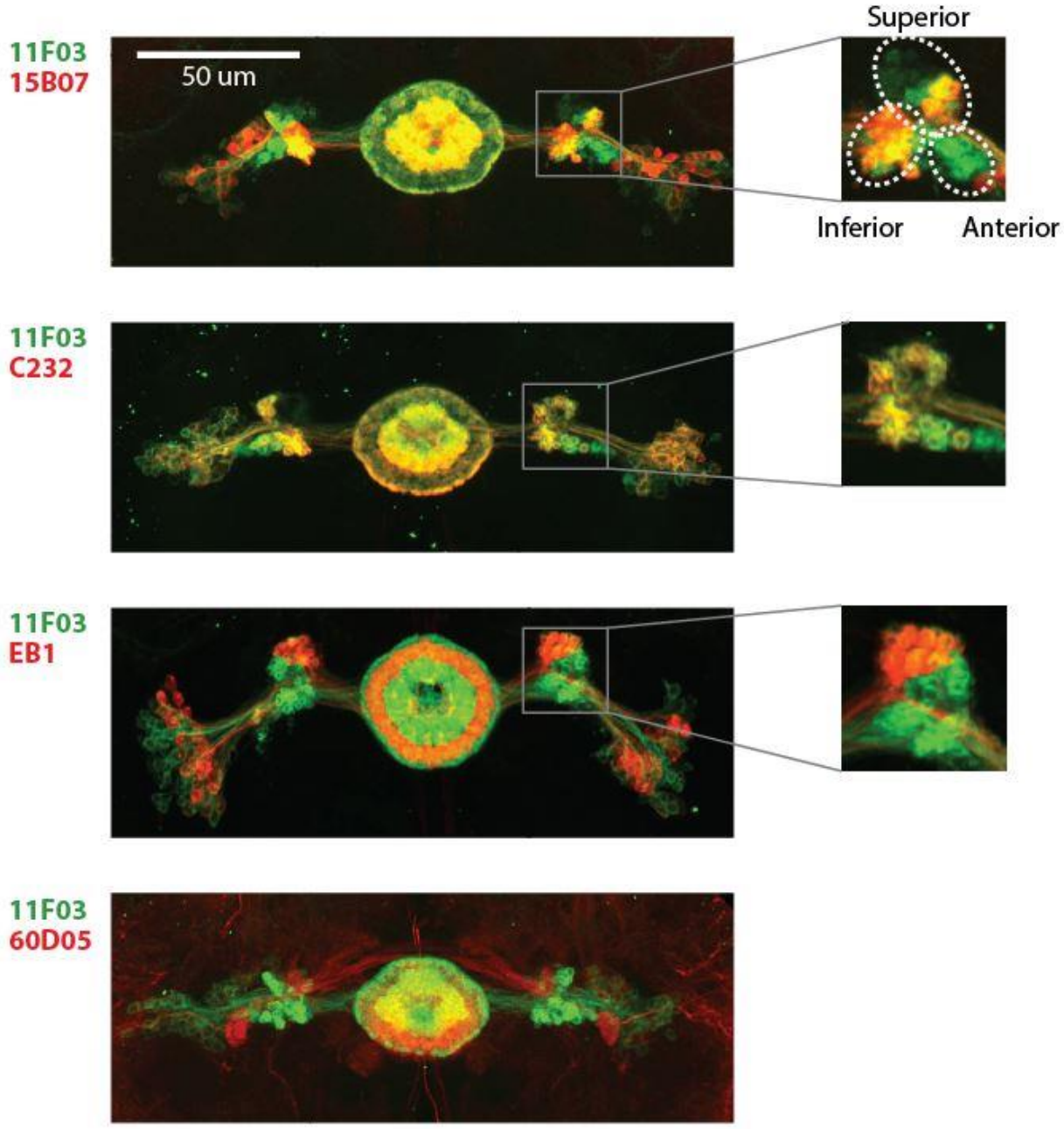
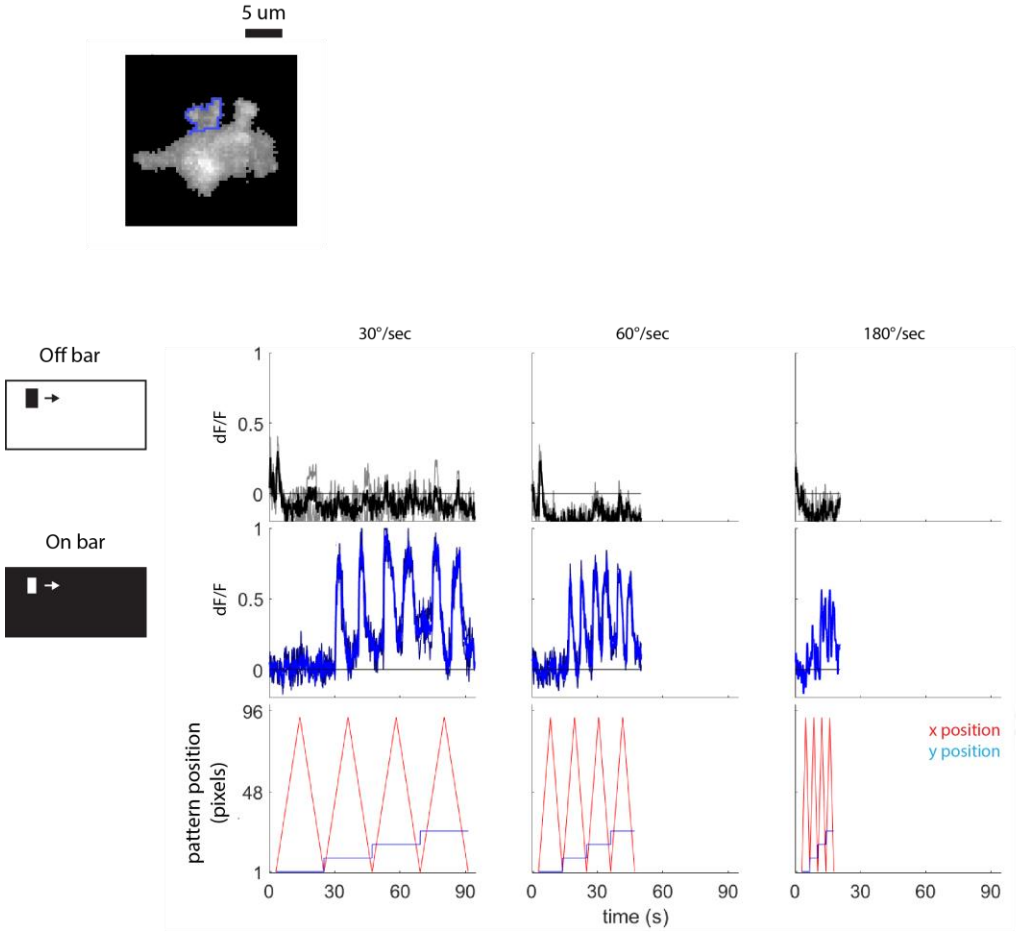


Figure 3.2: Relationship of 11F03 neurons to previously studied EB neurons. **(a)** 11F03 includes the expression of 15B07. 15B07 neurons have previously been shown to be required for visual place learning (Ofstad, *et al.*, 2011). **(b)** 11F03 and C232 (previously studied in Seelig & Jayaraman, 2013) have overlapping expression in superior and inferior lobes, but not in the inferior lobe. **(c)** EB1 and 11F03 co-label a small number of superior love neurons. **(d)** 60D05 (the famous tile neurons of Seelig & Jayaramn, 2015) and 11F03 do not overlap in the bulb; within the EB, wedge neurons are posterior to 11F03 ring neurons.

a


5 um
Off bar
On bar
30°/sec
60°/sec
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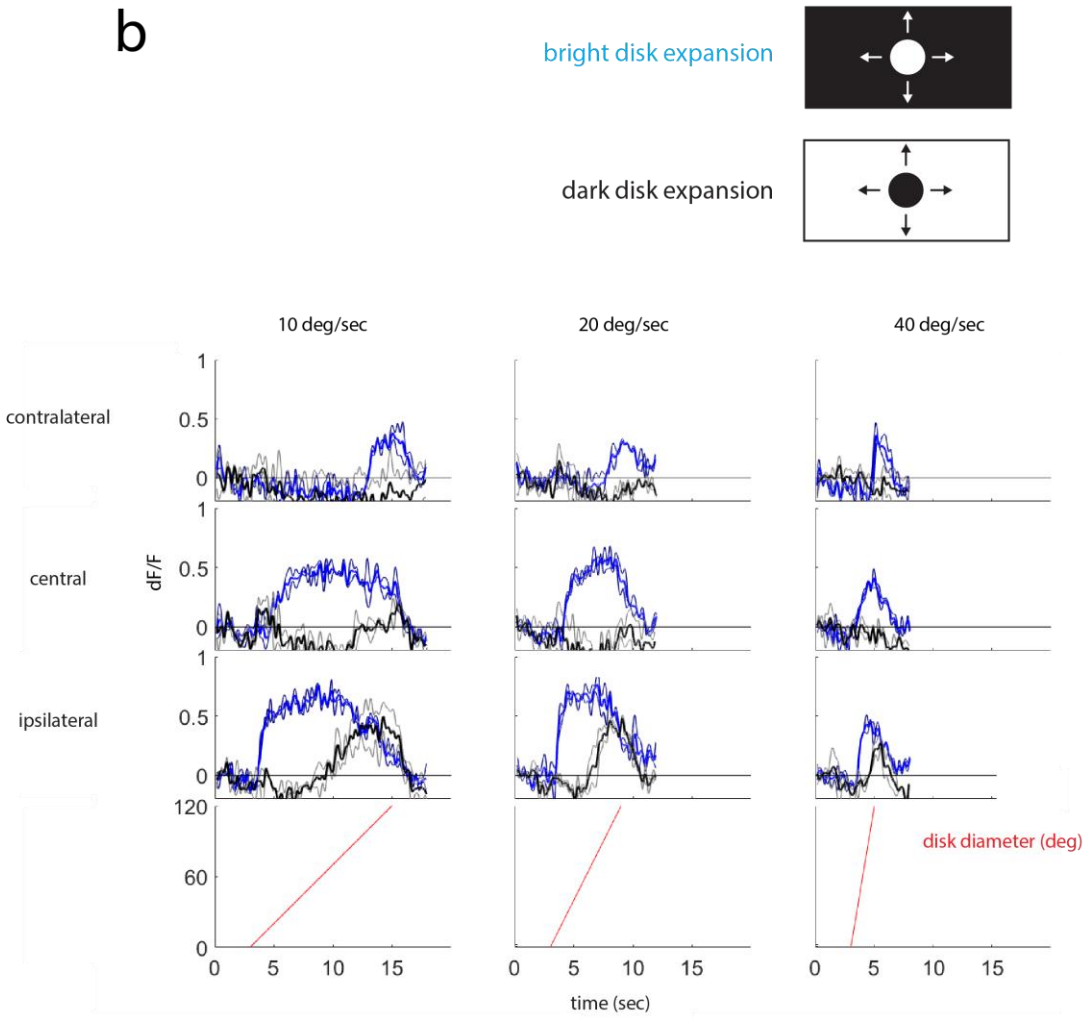
dF/F
pattern position (pixels)
x position
y position
time (s)


b
bright disk expansion
dark disk expansion
10 deg/sec
20 deg/sec
40 deg/sec
contralateral
central
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1
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time (sec)
disk diameter (deg)

C

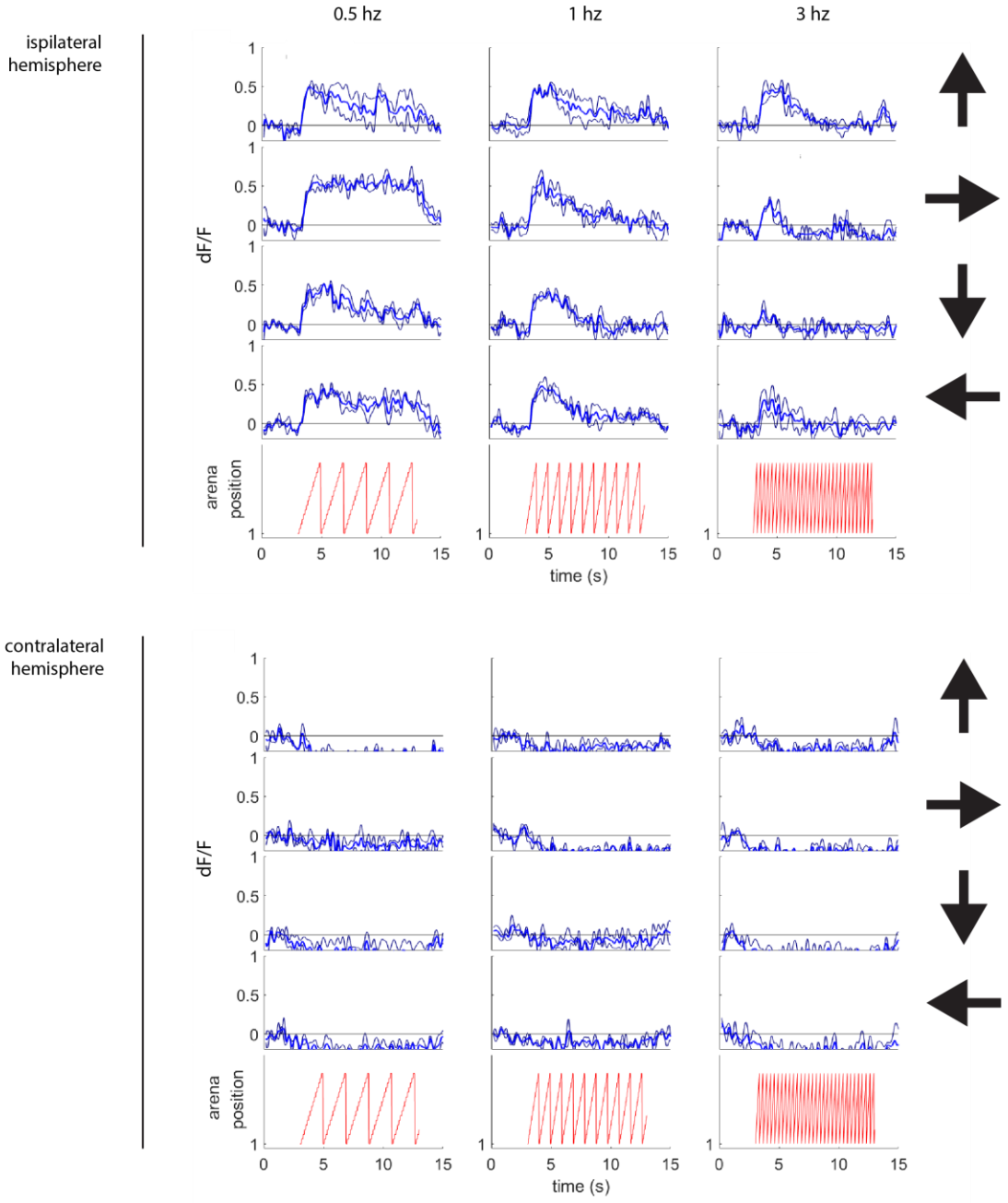


Figure 3.3 Example calcium responses to visual stimuli (a) 11F03 neurons respond strongly to small, bright bars (blue trace) but not dark bars (black trace). Responses are strongest at slowest speed (30°/second). **(b)** Responses to expanding disks. Neurons respond strongly to bright disk expansion within the ipsilateral visual field. To dark expanding disks, we observe early inhibition followed by late excitation at slow speeds. **(c)** Responses to

monocular presentation of moving square wave gratings (30° spatial frequency). Neurons respond to bars moving in the ipsilateral visual field, but do not display direction selectivity.

As in Omoto, *et al.*, we find evidence that bulb lobes constitute functionally distinct populations. Specifically, superior and anterior bulbs have stronger peak responses to small bright-object stimuli than inferior lobes. They also have clear on-excitatory receptive fields; inferior bulb neurons do not. Superior lobes have entirely ipsilateral receptive fields; anterior receptive fields are more distributed across the visual field with rare contralateral receptive fields. Conversely, inferior bulb responses are strongly correlated with fly movement, but not in superior and anterior lobes.

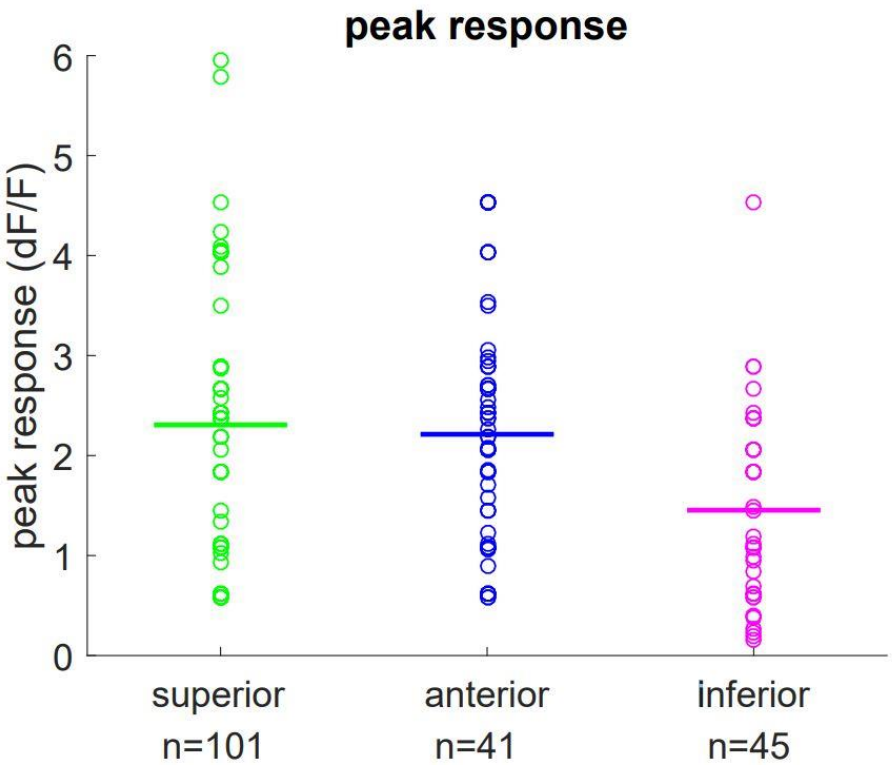


Figure 3.4 Response to small bright bar. During mapping trials, superior and anterior lobe neurons have larger peak responses to small bright bars.

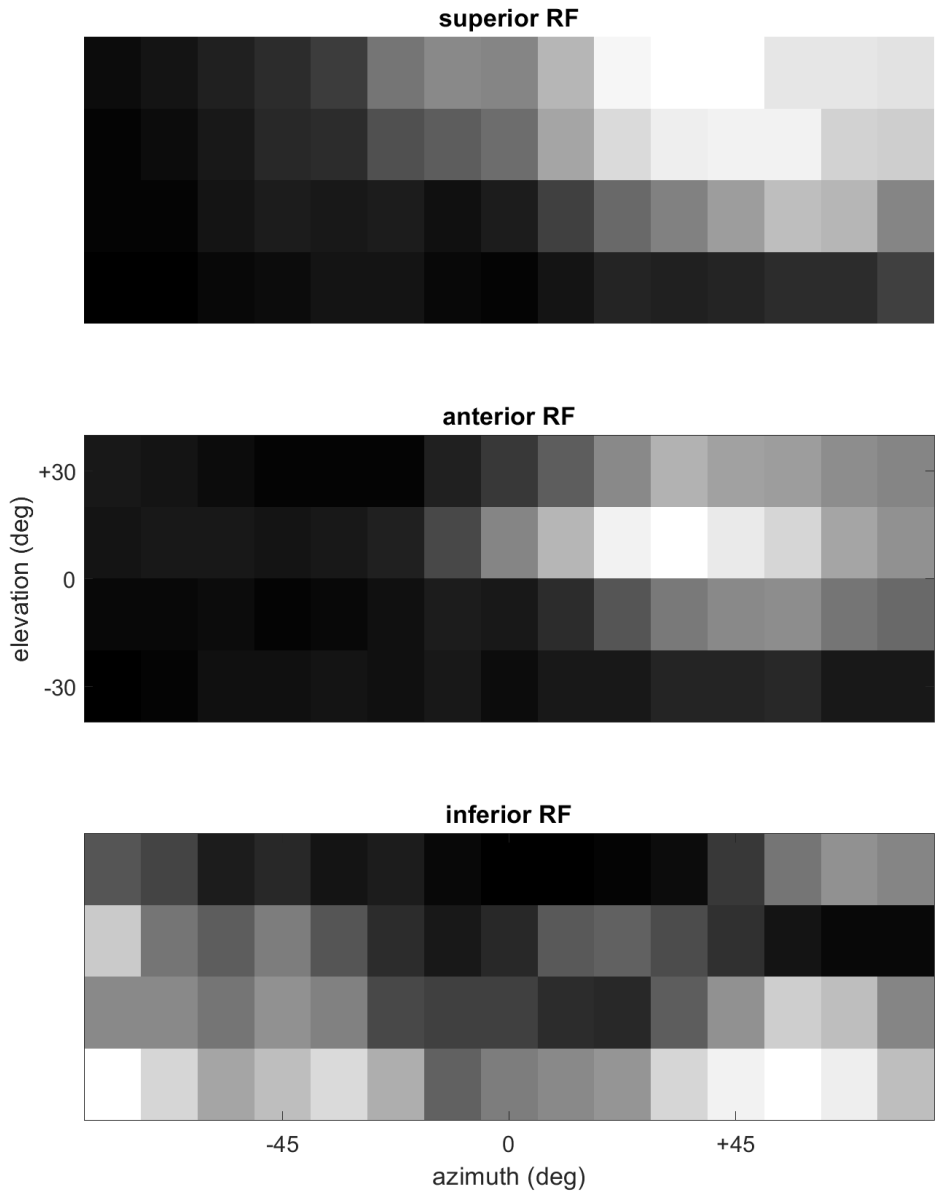


Figure 3.5 Example receptive fields. Superior and anterior lobe neurons have clear receptive fields; inferior lobe neurons do not.

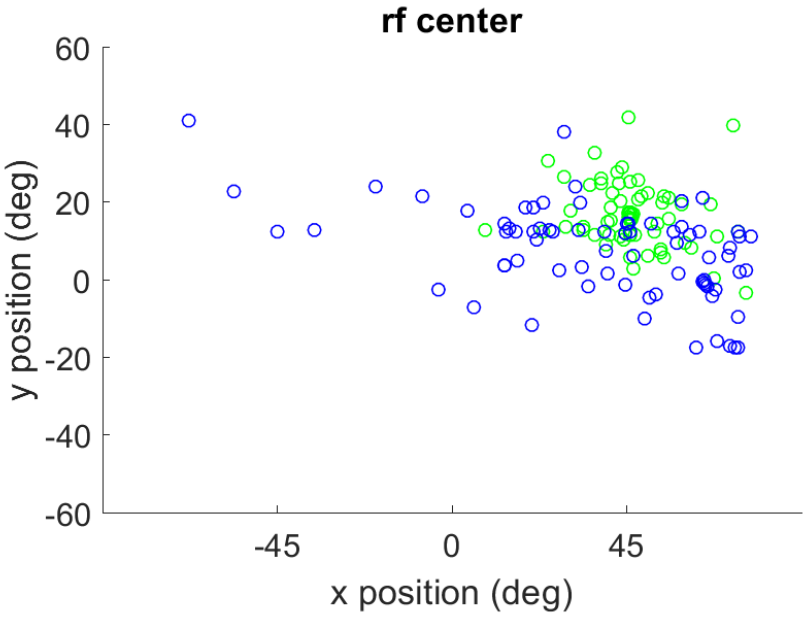


Figure 3.6 Receptive field location. Superior lobe neurons are restricted to the ipsilateral hemisphere; anterior lobe neurons are more distributed across the visual field

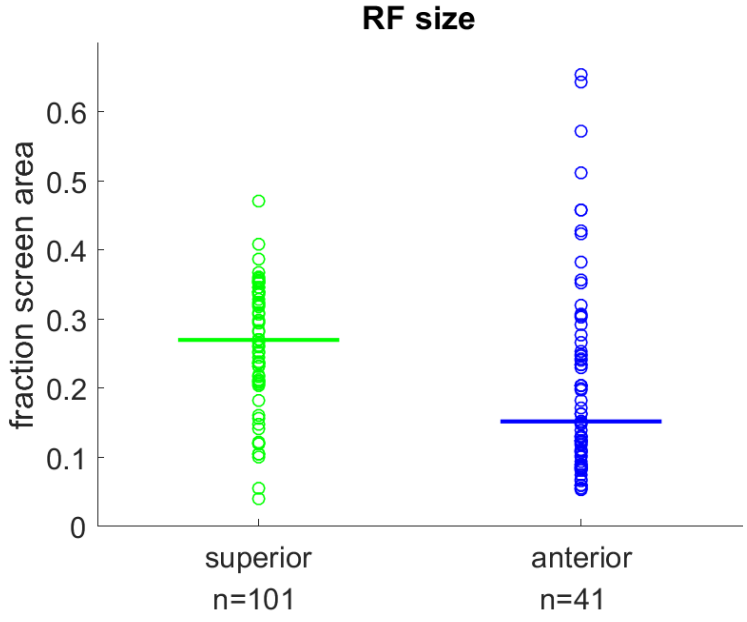


Figure 3.7 Receptive field size. Superior lobe receptive fields are larger than anterior lobe

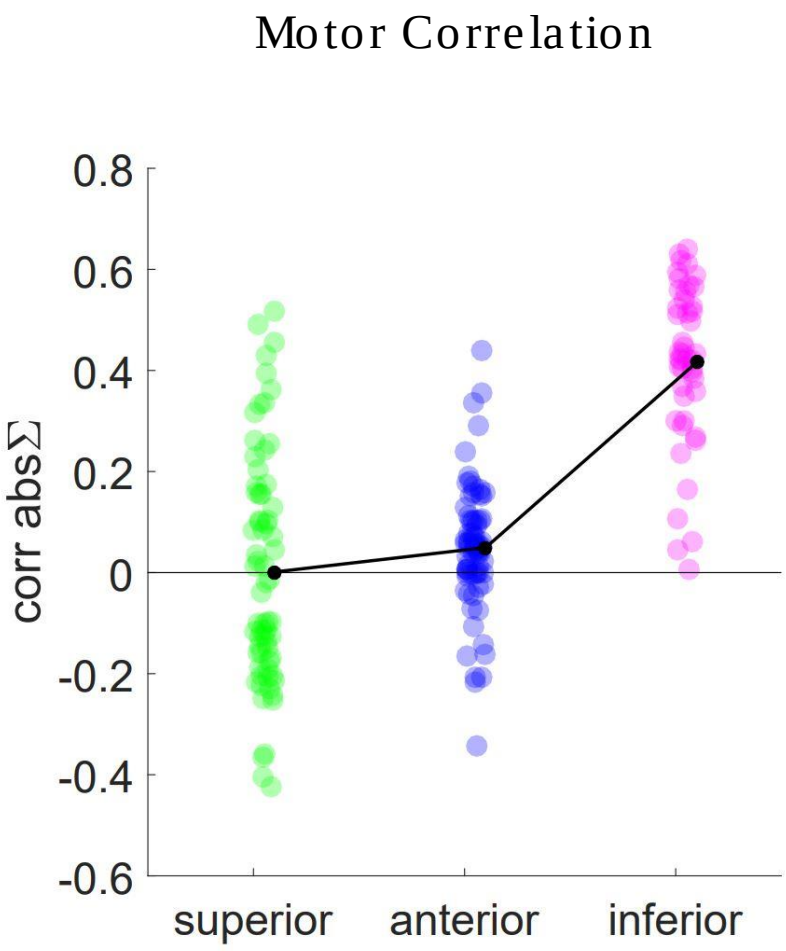


Figure 3.8 Motor correlations. Inferior lobe activity is tightly correlated with motor output. corr abs Σ = correlation with the sum of absolute ball rotation

Might these neurons convey temperature information as well? To look for temperature-related responses, we activated Hot Cells in the dark, and in the presence of visual stimuli, and recorded calcium responses in 11F03 dendrites. Remarkably, we observe near-complete inhibition of visual responses during Hot Cell stimulation. In the dark, we observe distinct "heat-off" responses to the cessation of Hot Cell stimulation. Clearly, these neurons are not purely visual; rather, they convey multisensory information from both the visual and thermosensory systems.

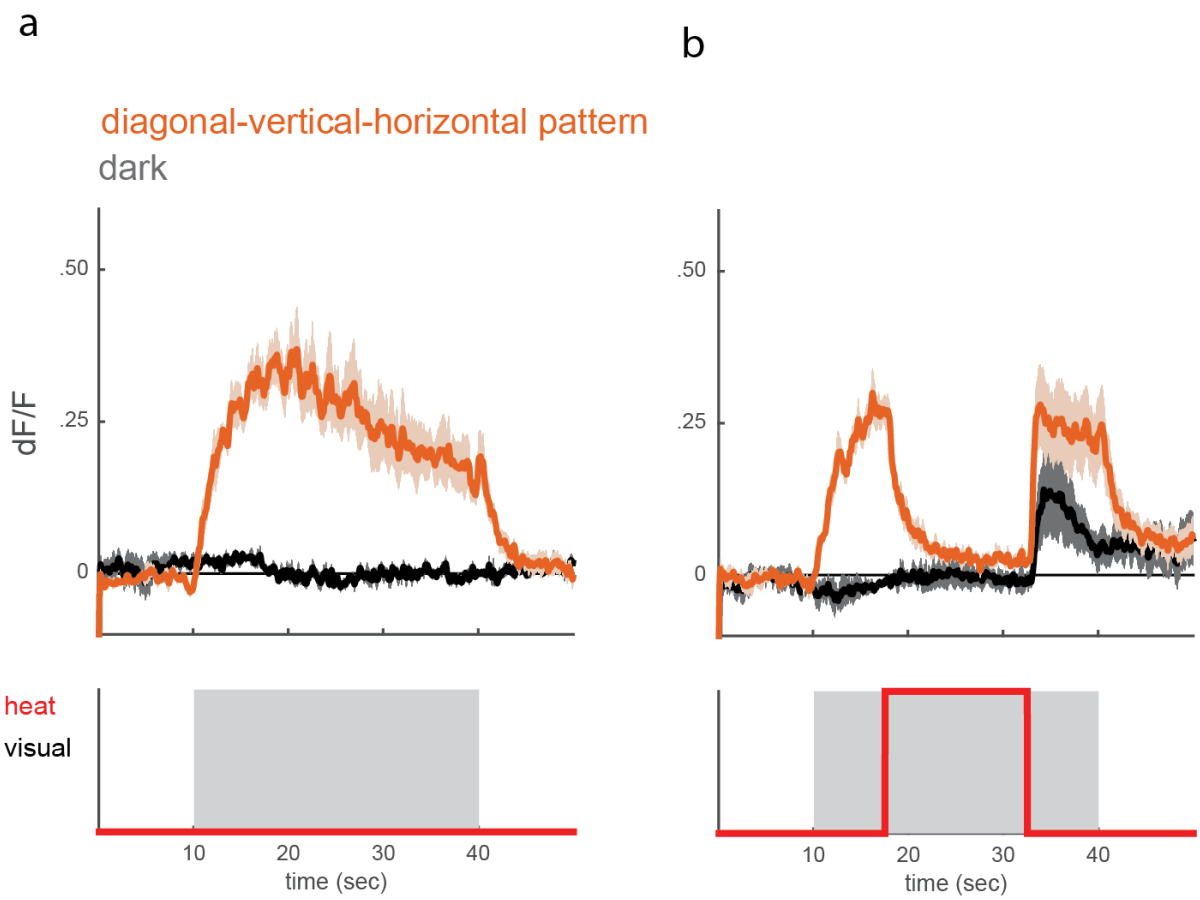


Figure 3.9 Visual responses are inhibited by Hot Cell activation (a) As in figure 3.2, 11F03 neurons respond to visual stimuli. The orange trace represents response to the stripes-bars-diagonal pattern used in closed-loop training The black trace shows activity (or lack thereof) when the fly is in the dark Grey box below shows the extent of visual stimulus presentation. **(b)** Pairing visual stimulus presentation with Hot Cell activation inhibits the visual response. In the dark, a distinct heat-off response is observed. Red trace represents the extent of Hot Cell stimulation.

These response properties (visually-sensitive, inhibited by virtual heat) make a prediction: 11F03 neurons should strongly respond when the fly reaches the cool zone during closed-loop learning behavior[2]. We examined calcium responses during training trials in our head-fixed learning assay. Indeed, we observe strong calcium responses in anterior lobe neurons (but not in other lobes) when the fly reaches the cool zone quadrant and escapes virtual heat.

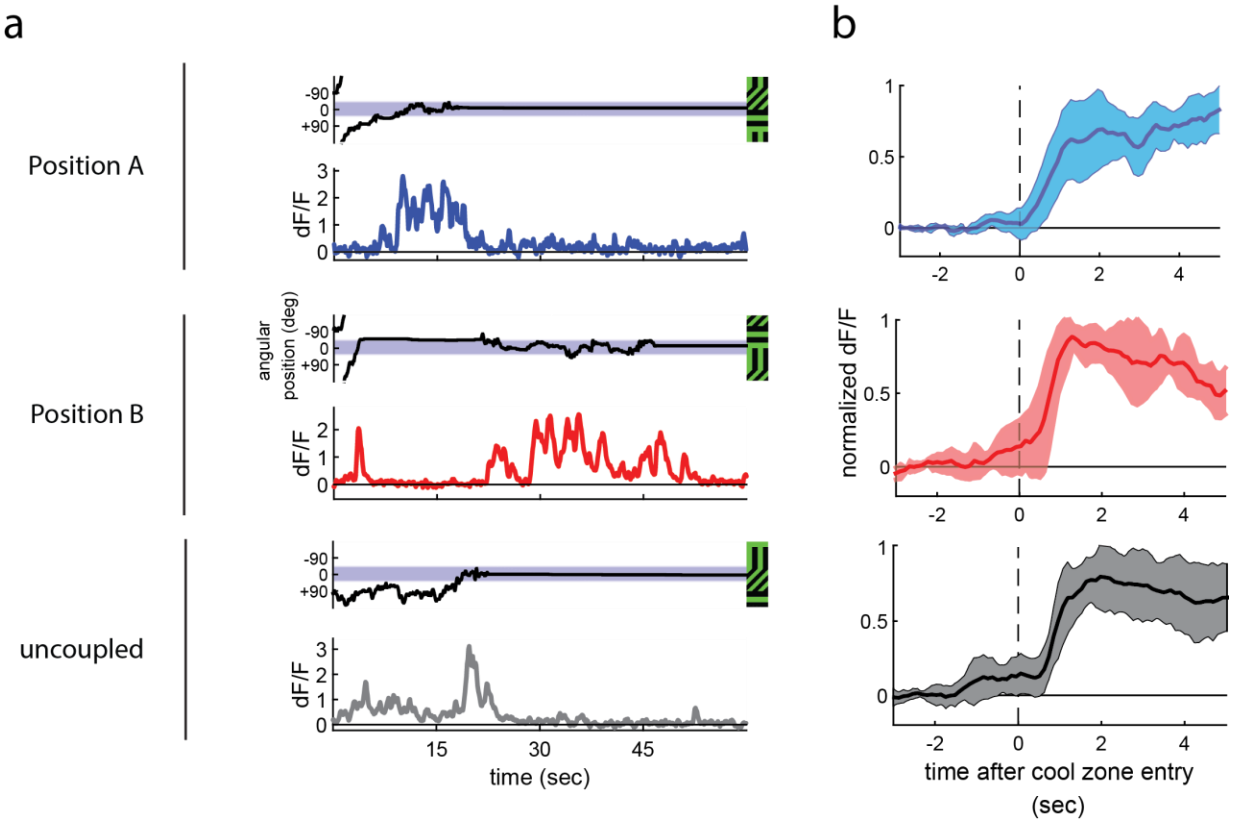


Figure 3.10 11F03 responses during closed-loop training trials (a) Single-trial behavior (above) and calcium traces (below) for Position A, Position B, and uncoupled control flies. Clear calcium responses are visible in anterior lobe neurons when the fly enters the cool zone. **(b)** Normalized, averaged responses of 11F03 neurons, aligned to cool zone entry. These responses are consistent across flies. (Position A n=6 flies; Position B n=7 flies; uncoupled control n=5)

[2] Details of this assay are discussed in Chapter 2.

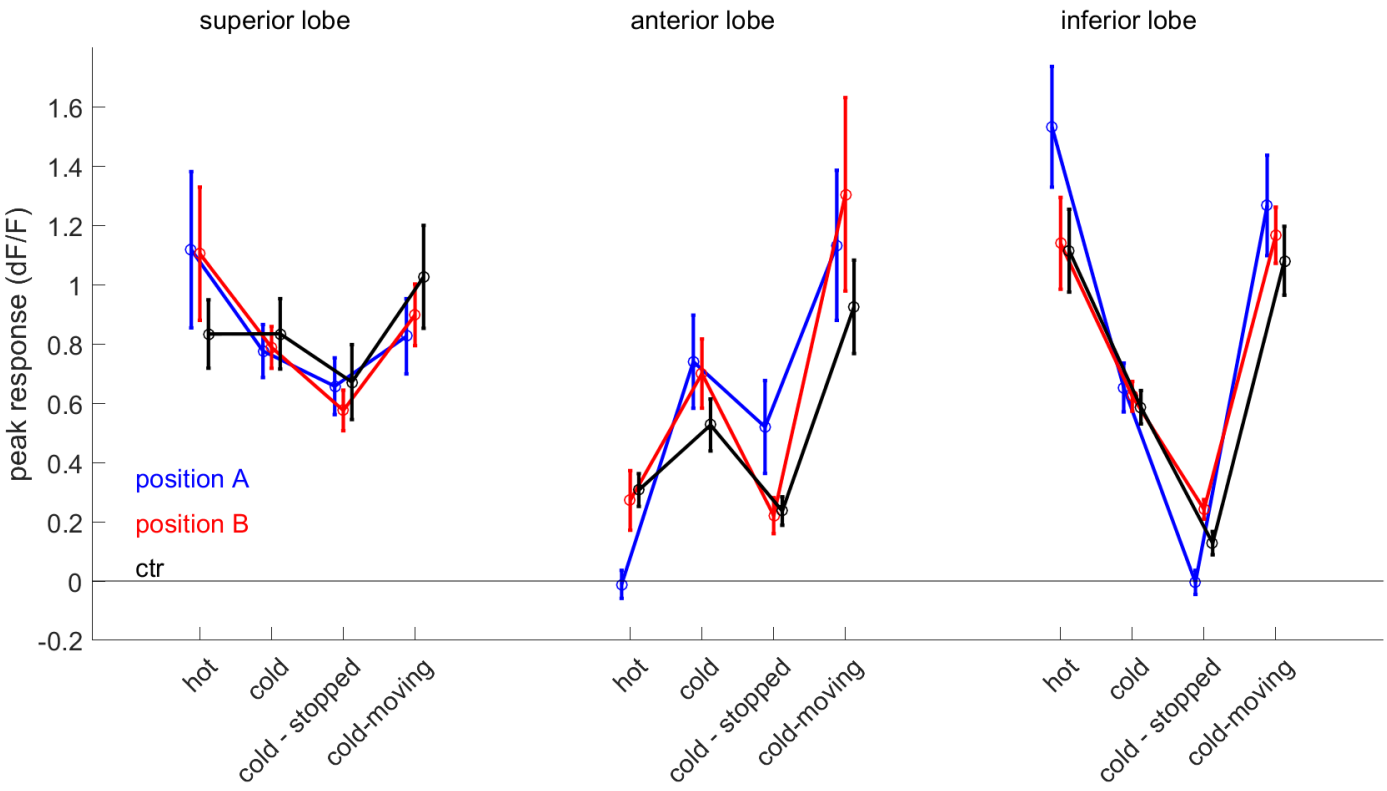


Figure 3.11 Activity modulation during closed-loop behavior. During learning trials, anterior lobe neurons are modulated by virtual heat and motion; inferior lobe neurons are modulated by moving. Superior lobe neuron activity is unaffected.

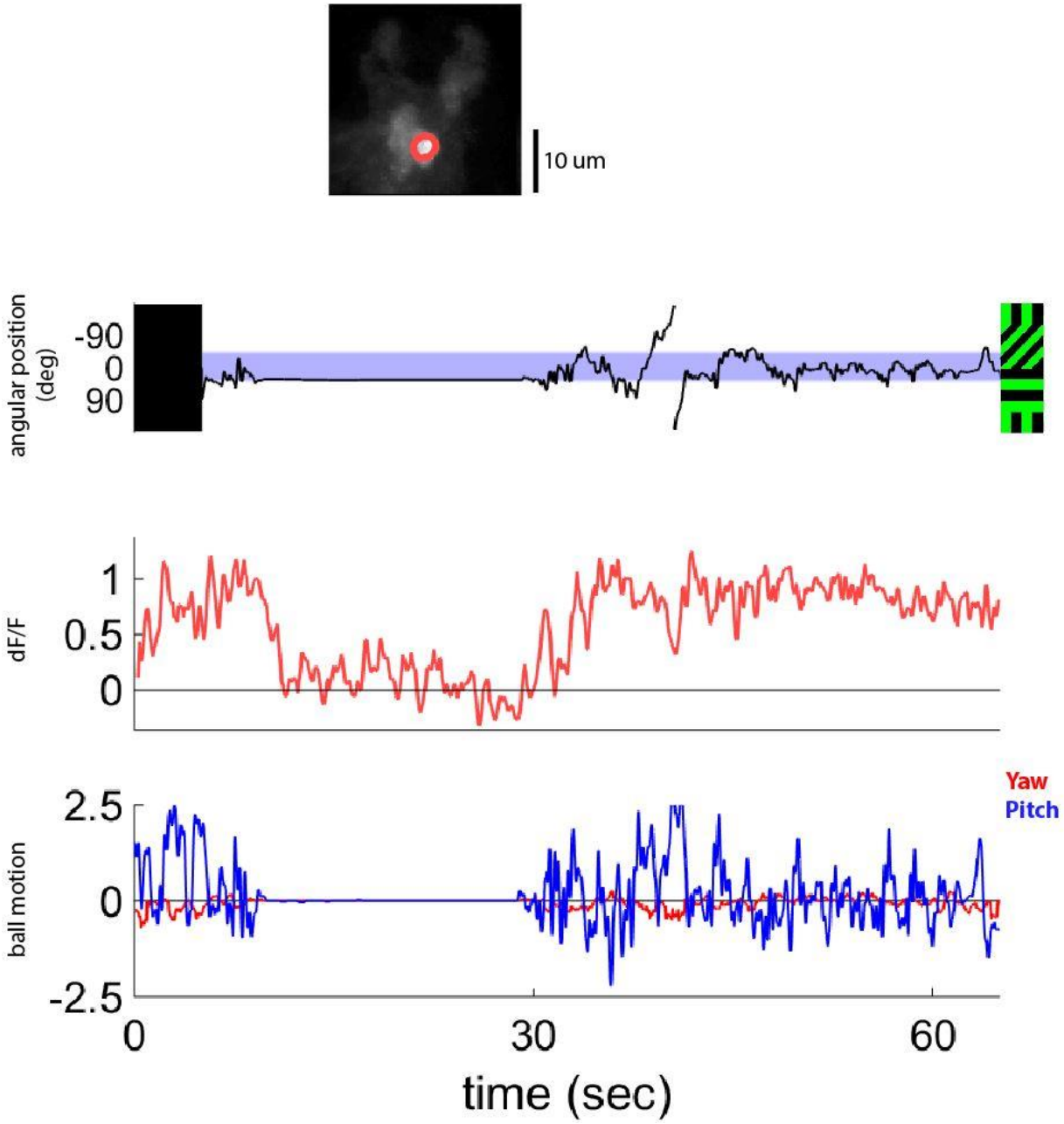


Figure 3.11 Inferior lobe neuron activity is highly correlated with fly motor output. An single-trial example of one such neuron.

A pressing question remains: does learning modify the response properties of these cells? An exciting possibility is that these neurons store visual features important for locating the rewarded quadrant. We examined calcium traces during the test trial of the visual learning experiment, but did not find evidence of preferential activity at the entrained orientation or changes in correlated activity with task parameters.

However, this type of experiment is not well suited to uncover subtle effects. In closed loop, each fly controls its own sensory experience. No two flies behave the same, making direct comparisons difficult. To address these problems, we designed an additional protocol to facilitate comparisons across flies while looking for learning-related activity changes. This protocol, and our findings, are described in the next chapter.

3.3 Methods

Fly stocks

Adult female flies were used 3-5 days post ecclosion. Full genotypes are as follows:

HC>Chr, 11F03>GCaMP6M: *w^+(DL)/w; 11F03-LexAp65(attP40)/HC-Gal4; UAS-CsChrimson-mCherry (su(Hw)attP1), pGP-JFRC59-13XLexAop2-IVS-p10-GCaMP6f (VK00005)/+*

HC>Chr, 11F03>GCaMP6f: *w^+(DL)/w; 11F03-LexAp65(attP40)/HC-Gal4; UAS-CsChrimson-mCherry (su(Hw)attP1), pGP-JFRC59-13XLexAop2-IVS-p10-GCaMP6f (VK00005)/+*

Imaging

Closed-loop behavioral experiments were conducted with the behavior platform described in Chapter 2. For open-loop characterization experiments, flies were cold-anesthetized, head-fixed and dissected under insect saline. The fly preparation is placed in front of 180° insect vision LED display, as described previously. The display is angled at 60° to compensate for the head tilt of the physiology preparation Stimulus design and control is performed in MATLAB. An optical fiber delivers light to the fly from a Thorlabs 660nm LED (Thorlabs M660F1). Flies are imaged with a Thorlabs Bergamo II 2-photon imaging system with a SpectraPhysics Mai-Tai DeepSee excitation laser source at 920 nm from 5-10 mW. Volumes of approximately 40 cubic microns are acquired in MATLAB at 5-10 hz.

Analysis

Analysis is performed by custom scripts written in MATLAB. Imaging volumes are collapsed to 2D by maximum intensity projection. Frames are motion-corrected, and regions of interest (ROIs) are hand drawn based on anatomy and pixel-wise variance. Baseline fluorescence is chosen by for each ROI by taking the 10^{th} percentile of pixel intensity values averaged within the ROI across all frames. Responses are reported as normalized change in pixel intensity (dF/F).

Chapter 4

Learning-related changes in ellipsoid body ring neurons

4.1 Background

Nearly all aspects of visual memory in *Drosophila* remain a mystery. What is known about learning and memory in flies comes mostly from work on olfactory memories. Significant progress has been made toward understanding olfactory memory in *Drosophila*. In our visual learning assay, we draw on the experience of the olfactory memory field while trying to address the limitations of previous studies.

The first definitive demonstration of learning and memory in *Drosophila* was reported in 1974, in an extraordinary paper from Seymour Benzer (Quinn, *et al.*, 1974). Benzer describes a strong capacity for flies to associate odor with punishment, and a relatively weak capacity for associating visual cues (in this case, color) with punishment. The odor assay, essentially unchanged for forty years, led to a massive body of work on olfactory memory in the *Drosophila* brain. Work on visual memory in *Drosophila* has proceeded in fits and starts.

Benzer was motivated to study the effect of genes on behavior. Olfactory learning was no exception. Perhaps due to his influence, the major preoccupation of odor memory studies in flies has been to identify genes and proteins involved in acquiring, storing, and reading out memory. Only recently have scientists turned to study the cellular components and synaptic activity changes associated with olfactory memory.

The "mushroom bodies," large paired fingerlike structures in the dorsal part of the fly brain, are the principal site of olfactory memory consolidation in *Drosophila* (McGuire, *et al.*, 2005). Within the mushroom bodies are neurons called Kenyon cells, which run the length of the mushroom body. Kenyon cells receive sparse input from the olfactory system via projection

neurons from the antennal lobe, the "smell center" of the fly brain. At their outputs, Kenyon cells synapse onto Mushroom Body Output Neurons (MBONs) within distinct compartments along the mushroom body. Different MBONs trigger aversion or attraction (Aso, *et al.,* 2014). It is thought that olfactory memories are stored by altering the strength of specific Kenyon-cell-to-MBON synapses. This allows the fly to flexibly remember a given odor as good (attractive) or bad (aversive), and respond accordingly.

While this is an appealing model, it has never been shown, in a behaving animal, that learning alters Kenyon-cell-to-MBON connections. Such an experiment is technically challenging. Three observations are necessary (but insufficient) to demonstrate learning-related change. These are:

1. **Behavioral change**: It is necessary to show that the animal has learned. Because we do not speak "fly," the animal demonstrating behavioral evidence of memory is the only way for the experimenter to ask if the animal has formed an association.
2. **Pre-training neural activity:** It is necessary to record the naïve response of neurons to the stimuli with which the animal will be trained, if any comparison is to be made.
3. **Post-training neural activity:** In an "expert" animal (verified by observing behavior), neural responses should change to the trained stimulus.

Two studies illustrate the limitations of previous efforts to observe memory-related activity change in *Drosophila*. Each is missing one key piece of evidence. In Wang, *et al.*¸ (2008), freewalking flies are trained in the traditional avoidance-learning odor assay. Expert flies are caught and prepared for imaging. Enhanced responses to the punished odor are observed in in the mushroom body, relative to an unpunished odor. However, without a baseline comparison of

the odor responses within a single brain, this study cannot observe change. Perhaps, the population of flies readily learning the association had better responses to the punished odor to begin with, and are therefore more able to learn.

Another more recent study observes robust alteration of responses, but lacks behavior. In Hige, *et al,* (2015), naïve responses of MBONs to odors are recorded. An odor is then paired with optogenetic activation of dopamine neurons, in such a pattern previously demonstrated to drive memory formation in other, free-walking flies. Dramatic suppression of MBON activity is observed following this pairing. While this is an elegant experiment, in the absence of behavior one cannot be certain if this change reflects real memory. In the design of our own experiment, we looked to address these limitations.

Significantly less is known about visual memory in *Drosophila* than olfactory memory. A targeted silencing screen in our own lab revealed that the ellipsoid body is required for visual place learning in *Drosophila* (Ofstad, *et al*., 2011). Strikingly, the mushroom bodies are not: silencing or even chemically ablating the mushroom bodies does not affect the ability of the fly to perform the place learning task. This demonstrates that the fly brain uses distinct anatomical substrates for different types of memory.

We designed a head-fixed virtual reality analog of the visual place learning task to study neural activity before, during, and after training. While we observe responses in ellipsoid body neurons during training behavior, we found no evidence for memory-related change from pre-training to test trials. This does not mean that no change has occurred. Two problems exist: first, pre-training and test trials are conducted in uniform virtual heat environments, to assay fly visual

preference in the absence of reward. We have shown that virtual heat inhibits visual responses in EB ring neurons; therefore, these trials are ill-suited to observe changes in activity.

Second, this experiment was performed in closed-loop. Using closed loop is necessary to allow the fly to behave naturalistically while head-fixed for imaging. During test trials, it allows the fly to reveal its learned association. However, closed-loop behavior gives the fly control over its own sensory experience. Each fly is different, making direct comparisons difficult across animals in the absence of a very large dataset. Moreover, the behavior of a trained fly is very different from its own behavior when it was naïve. To address these limitations while looking for changes in neural activity, we added standardized protocols to probe neural responses before and after closed-loop learning behavior.

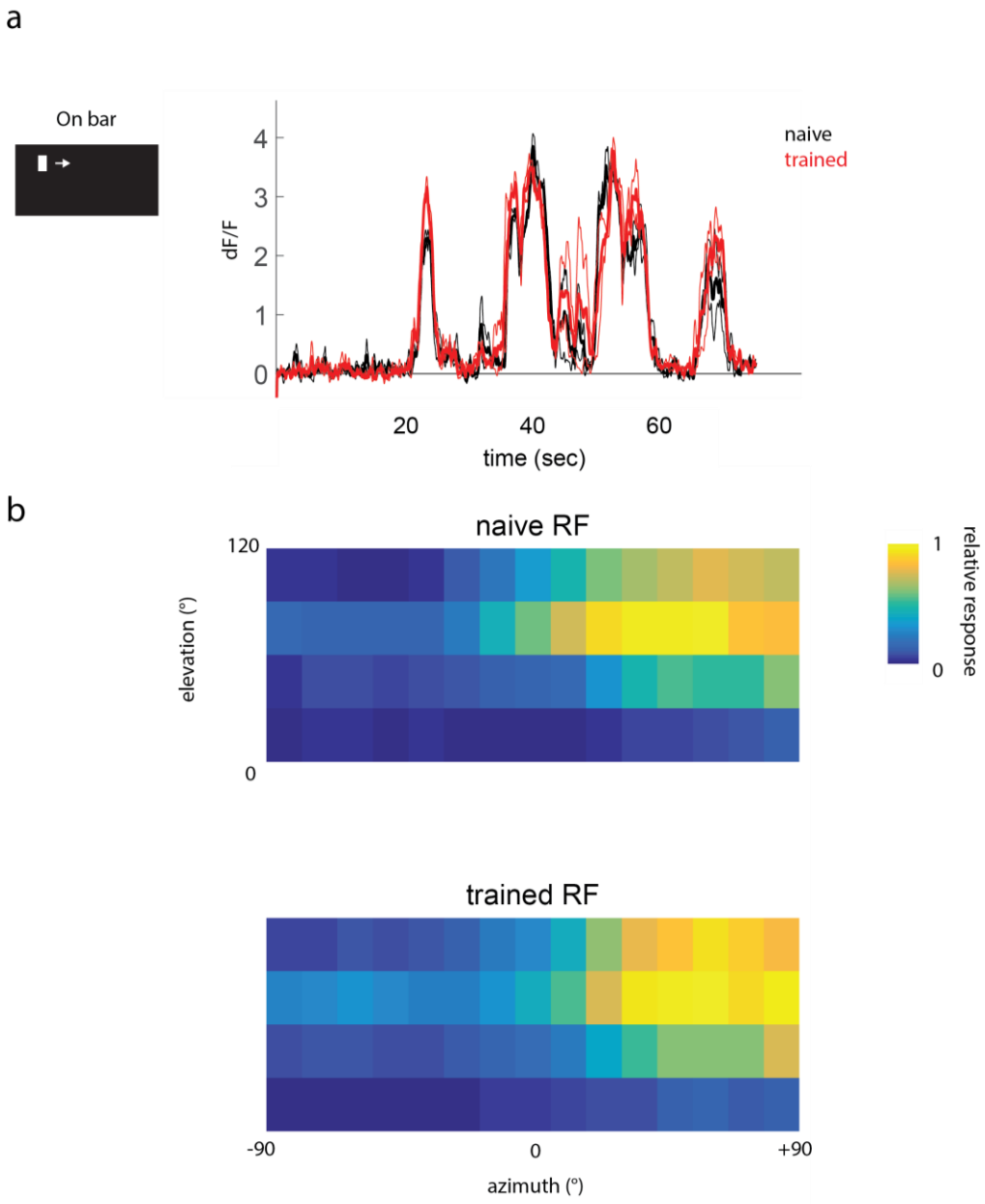
The design of this protocol was informed by the response properties of the EB ring neurons we characterized previously. Before training, we first characterize spatial receptive fields of 11F03 neurons by sweeping a small, bright bar horizontally across the visual field at different elevations. We then sweep the training pattern around the fly at 30° per second. Next, we repeat these pattern sweeps in the presence of virtual heat. Following training, we repeat all three characterization experiments. This stereotyped protocol facilitates direct comparisons within and across flies.

4.2 Results

Receptive field responses do not change from pre-training to post-training characterization. As seen in Figure 4.1, raw calcium responses to bar sweeps, and the

reconstructed receptive fields of the ROIs, remain identical. This result provides confidence that we analyze the same neuron throughout the characterization and closed-loop experiment phases.

Remarkably, responses to the paired presentation of the pattern plus virtual heat change dramatically. In naïve flies, virtual heat suppresses visual responses nearly completely. In trained flies, we observe partial recovery of the visual response. We observe no response recovery in



igure 4.1 Mapping receptive fields before and after closed-loop training (a) Calcium responses to small bar sweeps are nearly identical before (naive) and after (trained) closed-loop training. **(b**) Receptive fields reconstructed from raw calcium responses do not change.

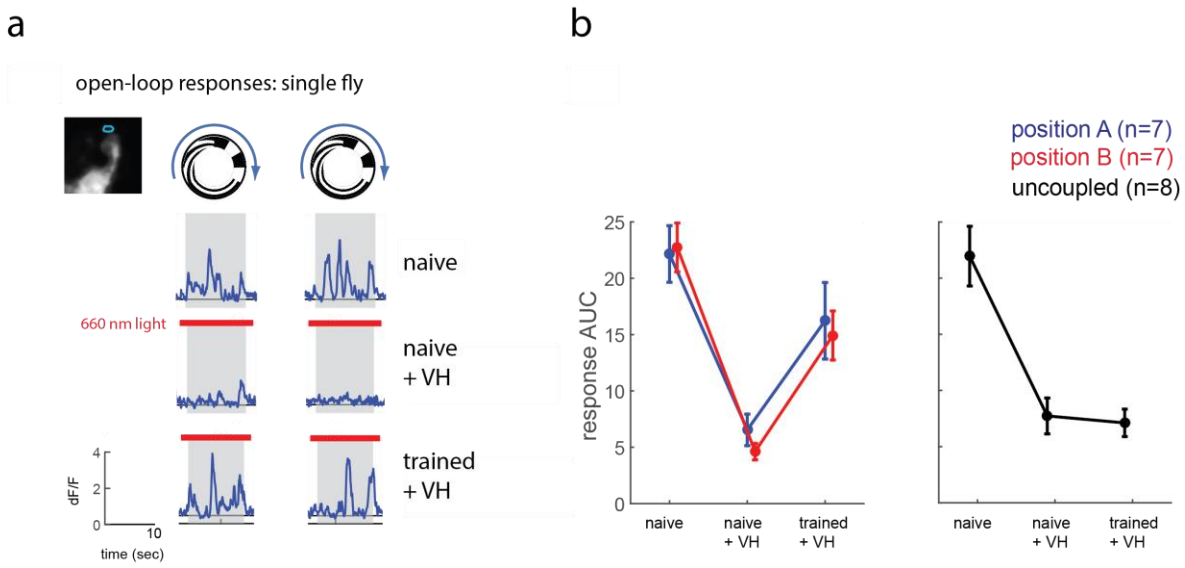


Figure 4.2 Visual responses recover after training (a) Single-trial responses to pattern sweeps within an individual animal. Before training, 11F03 neurons respond to pattern sweeps in the absence of Virtual Heat (VH). Virtual heat inhibits these responses before training. After training, responses recover. **(b)** Response recovery across animals. Integrated responses (area under curve, AUC) to naïve, naïve + Virtual Heat, and trained + Virtual Heat. Responses recover for both Position A and Position B flies, but not for uncoupled control flies.

uncoupled controls, which are trained without consistent visual cues. Clearly, this recovery is associated with learning.

What does this change represent? Is it a general effect of learning in the brain, or does it reflect the memory of a specific feature? To answer this question, we examined the change in responses as a function of pattern position, for both "Position A" flies (trained to prefer the seam between diagonal bars and vertical stripes), and "Position B" flies (trained to prefer the seam between vertical stripes and horizontal bars) at each location in the lobe.

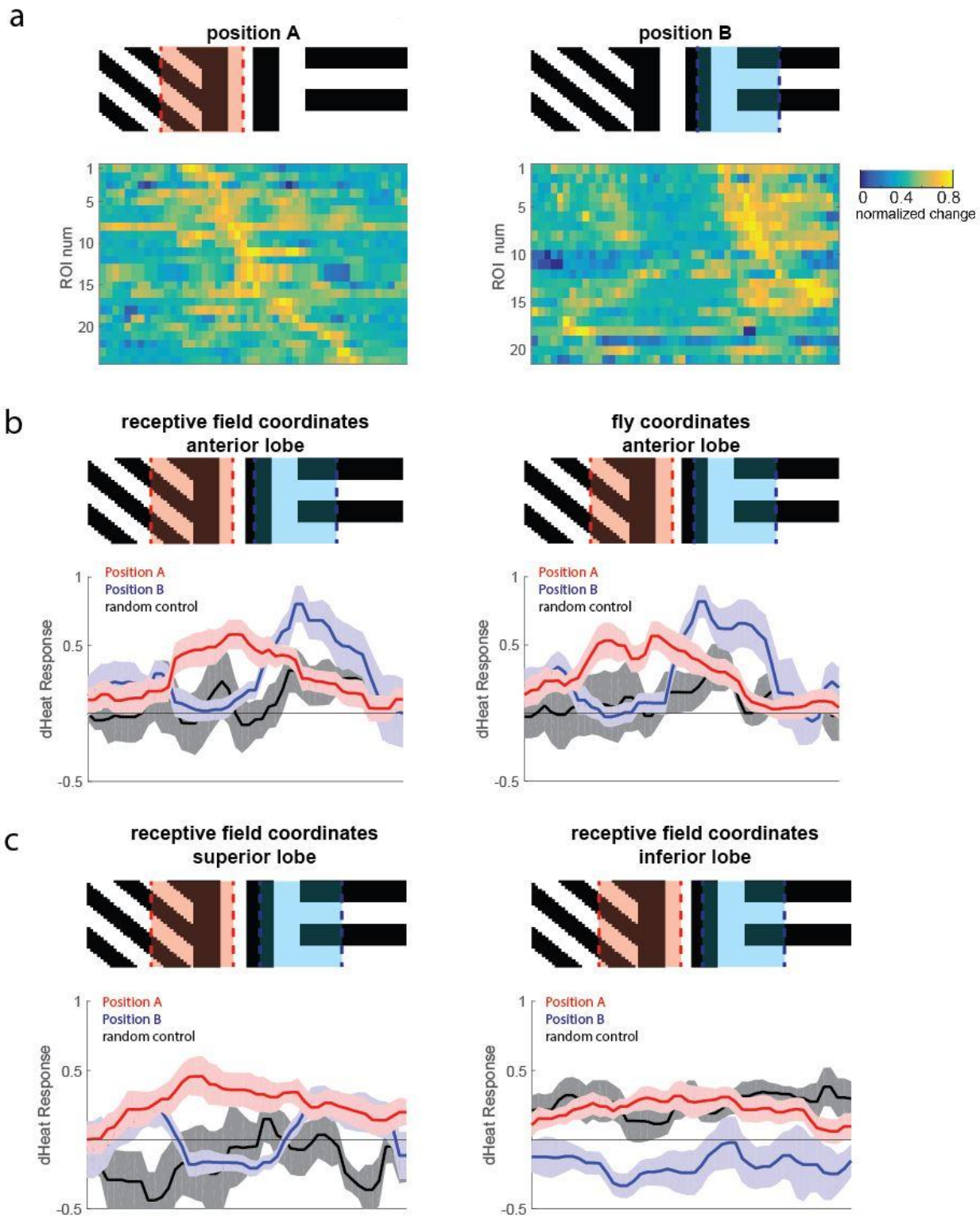

a
position A
position B
ROI num
1
5
10
15
20
0 0.4 0.8
normalized change
b
receptive field coordinates
anterior lobe
fly coordinates
anterior lobe
Position A
Position B
random control
dHeat Response
1
0.5
-0.5
c
receptive field coordinates
superior lobe
receptive field coordinates
inferior lobe

Figure 4.3 Disinhibition is specific to training condition (a) Heat map of the of response change to pattern +Virtual Heat from naïve to trained flies, plotted as a function of pattern position. Response recovery clusters to the right of the reward location. Each row represents a single fly. **(b)** Average of responses in (a), plus uncoupled condition, with and without receptive field coordinates. When plotted in fly coordinates (receptive field offset removed), it is clear these changes correspond to the learned location; most specifically to the edges of the cool zone. **(c)** These changes are lobe-specific: these changes are not observed in superior and inferior lobe neurons.

We observe clear structure in response disinhibition (Figure 4.3). This structure is distinct for trained visual features of Position A and Position B, and specific to anterior lobe neurons. Position A flies show a peak of response recovery to vertical stripes; Position B flies show a peak of similar magnitude to horizontal stripes. All imaging experiments were performed on the right ellipsoid body bulb. Therefore, this dis-inhibition is locked to the specific visual feature present within the receptive field of the neuron when the fly is oriented at the trained position.

Although this change is memory-specific, the possibility remains that it is an epiphenomenon of learning elsewhere in the brain. We wanted to see if 11F03 neurons are required for *remembering* visual features: if the changes we observe represent the visual memory, silencing these neurons during recall should abolish the learned preference. To test this, we designed a silencing experiment in which synaptic output is silenced only during the test phase of the closed loop experiment.

As seen in Figure 4.4, this is indeed the case. 11F03>Shibire^{TS} flies warmed to the restrictive temperature during testing demonstrate no visual preference, whereas 11F03>GCaMP flies are unaffected. This result shows that the synaptic output of 11F03 neurons is required during the recall phase of the experiment. Moreover, this demonstrates that the changes we observe in 11F03 neurons exist within cells critical for remembering learned visual cues.

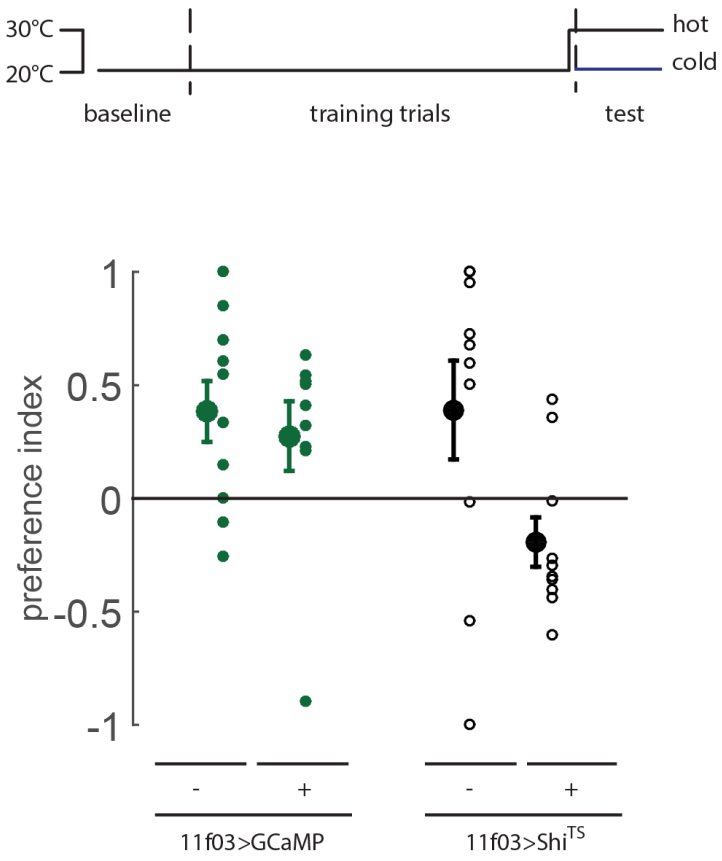


Figure 4.5 Silencing 11F03 neurons during test phase only abolishes memory. Flies expressing either temperature-sensitive Shibire, or GCaMP in 11F03 neurons are trained at room temperature, then tested at elevated temperature (+). Blocking 11F03 output during the recall phase abolishes the visual preference. (-) flies are room temperature controls.

4.3 Methods

Fly stocks

Adult female flies were used 3-5 days post ecclosion. Full genotypes are as follows:

HC>Chr, 11F03>GCaMP6f:

w^+(DL)/w; 11F03-LexAp65(attP40)/HC-Gal4; UAS-CsChrimson-mCherry (su(Hw)attP1), pGP-JFRC59-13XLexAop2-IVS-p10-GCaMP6f (VK00005)/+

HC>Chr, 11F03>Shi^{TS}:

w^+(DL)/w; 11F03-LexAp65(attP40)/HC-Gal4; UAS-CsChrimson-mCherry (su(Hw)attP1)/ pJFRC104-13XLexAop2-IVS-Syn21-Shibire^{ts1}-p10 (su(Hw)attP1)

Characterization Stimuli

Stimuli were delivered to the fly before and immediately following closed-loop training. Receptive fields were mapped by sweeping small bars (11.25° by 18.75°) horizontally across the visual field at non-overlapping elevations. Responses to the training pattern were tested by sweeping the pattern 360° in front of the fly at 30°/sec. Pattern presentation was repeated, paired with HC activation. Following training, this stimulus protocol was repeated in reverse order.

Imaging

Flies were cold-anesthetized, head-fixed and dissected under insect saline. The fly preparation is placed in front of 180° insect vision LED display, as described previously. The display is angled at 60° to compensate for the head tilt of the physiology preparation. Stimulus

design and control is performed in MATLAB. An optical fiber delivers light to the fly from a Thorlabs 660nm LED (Thorlabs M660F1). Flies are imaged with a Thorlabs Bergamo II 2-photon imaging system with a SpectraPhysics Mai-Tai DeepSee excitation laser source at 920 nm from 5-10 mW. Volumes of approximately 40 cubic microns are acquired in MATLAB at 5-10 hz.

Analysis

Analysis is performed by custom scripts written in MATLAB. Imaging volumes are collapsed to 2D by maximum intensity projection. Frames are motion-corrected, and regions of interest (ROIs) are hand drawn based on anatomy and pixel-wise variance. Baseline fluorescence is chosen by for each ROI by taking the 10^{th} percentile of pixel intensity values averaged within the ROI across all frames. Responses are reported as normalized change in pixel intensity (dF/F).

Silencing experiments

Silencing experiments were conducted with un-dissected flies head-fixed to the physiology shim. To heat the fly head, warm water was flowed through the saline reservoir using a liquid temperature controller (Warner Instruments CL-100). Heating was calibrated with thermal imaging. All flies were trained at 20°C (permissive temperature, not silencing). Flies were tested at either 20°C or 28°C (restrictive temperature for silencing).

Chapter 5
Discussion

Visual learning in *Drosophila* leads to selective disinhibition of visual responses. This disinhibition is strongest for visual features which reside within the receptive field of the neuron when the fly faces the reinforced orientation. We have shown that these changes occur in neurons critical for recall of this orientation. This result suggests a specific role for ellipsoid body neurons during memory-guided navigation: these neurons selectively and flexibly recognize features of the environment predictive of reward.

Several questions remain. First, what mechanisms could account for this change? A few possibilities exist. Learning could weaken thermal inhibition of visual responses. Alternatively, visual responses could be strengthened, overcoming inhibition by brute force. Strengthening visual responses could occur in one of two ways. Visual responses could be globally enhanced by "dialing up" the output of EB ring neurons. Instead, the filter imposed on visual space by these neurons could change in such a way as to respond more strongly to specific pattern features. Because the change we observe in EB ring neurons is quite specific to the training position, our data is most consistent with this last possibility.

What is the downstream consequence of this change? Our data suggest 11F03 ring neurons are move active in aversive thermal environments after training than before training. What, then, is the functional role of EB ring neurons within the ellipsoid body circuit? The ellipsoid body has been shown to be critical for maintaining the fly's heading toward visual landmarks (Neuser *et al.,* 2008). Perhaps 11F03 neurons influence the turning behavior of the fly, either by triggering turning toward learned visual features, or suppressing turning when the fly is oriented toward them. To investigate this possibility, one could activate 11F03 neurons and observe the effect on turning behavior.

Finally, why does Hot Cell activation inhibit visual responses? The benefits of this are not immediately clear. One possibility is that 11F03 neurons are valence-sensitive visual neurons, and respond to visual cues only in the absence of aversive stimuli. If this is true, the visual responses of 11F03 neurons should be inhibited by aversive stimuli other than heat: bitter taste, perhaps; or electric shock. Context-dependent visual responses would have obvious utility within a navigation circuit.

In this dissertation, I have described our efforts to study changes in neural activity associated with visual learning in the fly. Toward this goal, we have made several important developments. We have shown that activating heat sensors directly, rather than using "real" heat, is sufficient to drive avoidance and learning in *Drosophila*. We have observed cross-modal interactions between vision and heat within single neurons in the *Drosophila* central complex, demonstrating that the ellipsoid body receives information from multiple sensory streams. We have developed a novel visual learning assay, compatible with imaging, to study neural activity before, during, and after learning within single brains. We have observed activity changes accompanying visual learning within a navigation center of the fly brain. And, we have confirmed that these same neurons are necessary for remembering visual features.

Drosophila are capable of complex, nuanced behaviors – achieved with a compact brain that is convenient for study. The development of a head-fixed visual learning assay, compatible with imaging, adds the ability to monitor brain activity during learning behavior to the substantial tools available to probe neural circuits in *Drosophila.* While we focused our study on the ellipsoid body, changes are likely to be observed elsewhere in the *Drosophila* central complex. For example, the fan-shaped body, a layered structure immediately dorsal and posterior

to the ellipsoid body, has also been implicated in visual memory in *Drosophila* - specifically in the fly's ability to remember orientations and shapes (Liu et al. 2006). It would be interesting to compare learning-related activity changes in the fan-shaped body to those observed in the ellipsoid body. Does the fan-shaped body receive multisensory information as well, and how do the rules for integration compare to the heat-mediated inhibition we have seen? Moreover, using this platform with flies behaving in virtual odor environments, rather than visual ones, would be a useful technique to study olfactory memory at the KC-MBON synapse. Experiments like these should provide powerful insight into how the brains of behaving animals develop and read out memory.

References

Al-Anzi, B., Tracey, W.D., Benzer, S. (2006). Response of *Drosophila* to wasabi is mediated by *painless*, the fly homolog of mammalian TRPA1/ANKTM1. *Current Biology*. 16, 1034-1040.

Altner, H., Loftus, R. (1985). Ultrastructure and function of insect thermo- and hygroreceptors. *Annual Reviews Entomology*. 30, 273-295.

Aso, Y., Sitaraman, D., Ichinose, T., Kaun, K. R., Vogt, K., Belliart-Guérin, G., & Rowell, W. J. (2014). Mushroom body output neurons encode valence and guide memory-based action selection in Drosophila. *Elife*, *3*, e04580.

Bahl, A., Ammer, G., Schilling, T., Borst, A. (2013). Object tracking in motion-blind flies. *Nature Neuroscience.* 16, 730-738.

Buchner, E. (1976). Elementary movement detectors in an insect visual-system. *Biol. Cybern.* 24, 85–101

Chiappe, M. E., Seelig, J. D., Reiser, M. B., & Jayaraman, V. (2010). Walking modulates speed sensitivity in Drosophila motion vision. *Current Biology*, *20*(16), 1470-1475.

Chen T, Wardill TJ., Sun Y, Pulvar SR., Renninger SL., Baohan A, Schreiter ER, Kerr RA, Orger MB., Jayaraman V, Looger LL, Svoboda K, Kim DS. (2013). Ultrasensitive fluorescent proteins for imaging neuronal activity. *Nature* 499, 2950300.

Dombeck, D., Harvey, C., Tian, L., Looger, L., Tank, D. (2010). Functional imaging of hippocampal place cells at cellular resolution during virtual navigation. *Nature Neuroscience.* 13, 1433-1440.

Dubnau, J., Grady, L., Kitamoto, T., & Tully, T. (2001). Disruption of neurotransmission in Drosophila mushroom body blocks retrieval but not acquisition of memory. *Nature*, *411*(6836), 476-480.

Duistermars, B. J., & Frye, M. A. (2010). Multisensory integration for odor tracking by flying Drosophila: behavior, circuits and speculation. *Communicative & integrative biology*, *3*(1), 60-63.

Foelix, R.F., Stocker, R.F., Steinbrecht, R.A. (1989). Fine structure of a sensory organ in the arista of *Drosophila melanogaster* and some other dipterans. *Cell and Tissue Research.* 258, 277-287.

Frank, D. D., Jouandet, G. C., Kearny, P. J., Macpherson, L. J. & Gallio, M. (2015) *Nature* 519, 358–361.

Gallio, M., Ostad, T.A., Macpherson, L.J., Wang, J.W., Zuker, C.S. (2011). The coding of temperature in the *Drosophila* brain. *Cell.* 144, 614-624.

Gangola, P., Pant, H. (1983). Temperature dependent conformational changes in calmodulin. *Biochemical and Biophysical Research Communications*. 111 (1), 301-305.

Hamada F.N., Rosenzweig, R., Kang, K., Pulver, S., Ghezzi, A., Jegla, T.J., Garrity, P.A. (2008). An internal thermal sensor controlling temperature preference in *Drosophila*. *Nature*. 454 (10), 217-222.

Hanesch, U., Fischbach, K. F., & Heisenberg, M. (1989). Neuronal architecture of the central complex in Drosophila melanogaster. *Cell and Tissue Research*, *257*(2), 343-366.

Heinze, S. and U. Homberg (2007). "Maplike representation of celestial E-vector orientations in the brain of an insect." Science 315(5814): 995-7.

Heinze, S. and S. M. Reppert (2011). "Sun compass integration of skylight cues in migratory monarch butterflies." Neuron 69(2): 345-58.

Hige T, Aso Y, Modi M, Rubin GM, Turner GC. (2015) Heterosynaptic plasticity underlies aversive olfactory learning in Drosophila. *Neuron*. 88 (5), 985-98.

Jenett, A., Rubin, G.M., Ngo, T.-T.B., Shepherd, D., Murphy, C., Dionne, H., Pfeiffer, B.D., Cavallaro, A., Hall, D., Jeter, J., et al. (2012). A GAL4-driver line resource for Drosophila neurobiology. Cell Rep. 2, 991–1001

Klapoetke, N.C., Murata, Y., Kim, S.S., Pulver, S.R., Birdsey-Benson, A., Cho, Y.K., Morimoto, T.K., Chuong, A.S., Carpenter, E.J., Tian, Z., Wang, J., Xie, Y., Yan, Z., Zhang, Y., Chow, B.Y., Surek, B., Melkonian, M., Jayaraman, V., Constantine-Paton, M., Wong, G.K., Boyden, E.S. (2014). Independent optical excitation of distinct neural populations. *Nature Methods.* 3, 338-346.

Kim, A. J., Fenk, L. M., Lyu, C., & Maimon, G. (2017). Quantitative Predictions Orchestrate Visual Signaling in Drosophila. *Cell*.

Liu, G., Seiler, H., Wen., A. Zars, T. Ito, K., Wolf, R., Heisenberg, M., Liu, L. (2006). Distinct memory traces for two visual features in the *Drosophila* brain. *Nature*. 439, 551–556.

McGuire, S. E., Deshazer, M., & Davis, R. L. (2005). Thirty years of olfactory learning and memory research in Drosophila melanogaster. *Progress in neurobiology*, *76*(5), 328-347.

Maimon, G., Straw, A.D. & Dickinson, M.H. (2010). Active flight increases the gain of visual motion processing in *Drosophila* . Nat. Neurosci. 13, 393–399

Ni, L., Bronk, P., Chang, E.C., Lowell, A.M., Flam, J.O., Panzano, V.C., Theobald, D.L., Griffith, L.C., Garrity, P.A. (2013). A gustatory receptor paralogue controls rapid warmth avoidance in *Drosophila. Nature.* 500 (7464), 580-584.

Omoto, J., Keles, M., Nguyen, B., Bolanso, C., Lovick., J., Frye, M., Hartenstein, V. (2017). Visual input to the *Drosophila* central complex by developmentally and functionally distinct neuronal populations *Current Biology*. (27) 1098-1110.

Neuser, K., Triphan, T., Mronz, M., Poeck, B., & Strauss, R. (2008). Analysis of a spatial orientation memory in Drosophila. *Nature*, *453*(7199), 1244-1247.

Ofstad, T., Zuker, C., Reiser, M. (2011). Visual place learning in *Drosophila melanogaster. Nature*. 474, 204-207.

Poggio, T., Reichardt, W. (1976). Visual control of orientation behavior in the fly. *Quarterly Reviews of Biophysics*. 9 (3), 377-438.

Reiser, M.B. & Dickinson, M.H. (2008). A modular display system for insect behavioral neuroscience. J. Neurosci. Methods 167, 127–139

Rosenzweig, M., Brennan, K., Tayler, T., Phelps, P., Patapoutian, A., Garrity, P. (2005). The *Drosophila* ortholog of vertebrate TRPA1 regulates thermotaxis. *Genes & Development*. 19, 419-424.

Sayeed, O., Benzer, S. (1996). Behavioral genetics of thermosensation and hygrosensation in *Drosophila. Proceedings of the National Academy of Sciences*. 93, 6079-6084.

Sokabe, T., Tsujiuchi, S., Kadowaki, T., Tominaga, M. (2008). *Drosophila* painless is a Ca^{2+} -requiring channel activated by noxious heat. *The Journal of Neuroscience*. 28 (40), 9929-9938.

Tang, X., Platt, M.D., Lagnese, C.M., Leslie, J.R., Hamada, F.N. (2013). Temperature integration at the AC thermosensory neurons in *Drosophila*. *The Journal of Neuroscience*. 33 (3), 894-901.

Tracey, W.D., Wilson, R.I., Laurent, G., Benzer, S. (2003). *painless*, a *Drosophila* gene essential for nociception. *Cell*. 113, 261-273.

Seelig, J., Chiappe, ME., Lott, G., Dutta, A., Osborne, E., Reiser, M., Jayaraman, V. (2010). Two-photon calcium imaging from head-fixed *Drosophila* during optomotor walking behavior. *Nature Methods*. 7, 535-540.

Seelig, J., Jayaraman, V. (2013) Feature detection and orientation tuning in the Drosophila central complex.. *Nature* 503, 262-266.

Seelig, J., Jayaraman, V. (2015) Neural dynamics for landmark orientation and angular path

integration. *Nature* 521, 186-191.

Strother, J. A., Nern, A., & Reiser, M. B. (2014). Direct observation of ON and OFF pathways in the Drosophila visual system. *Current Biology*, *24*(9), 976-983.

Taube JS, Muller RU, Ranck JB Jr (1990) Head-direction cells recorded from the postsubiculum in freely moving rats. I. Description and quantitative analysis. *Journal of Neuroscience.* 10:420–435.

Weir, P., Dickinson, M. (2015) Functional divisions for visual processing in the central brain of flying *Drosophila*. *PNAS*. 112 (40)

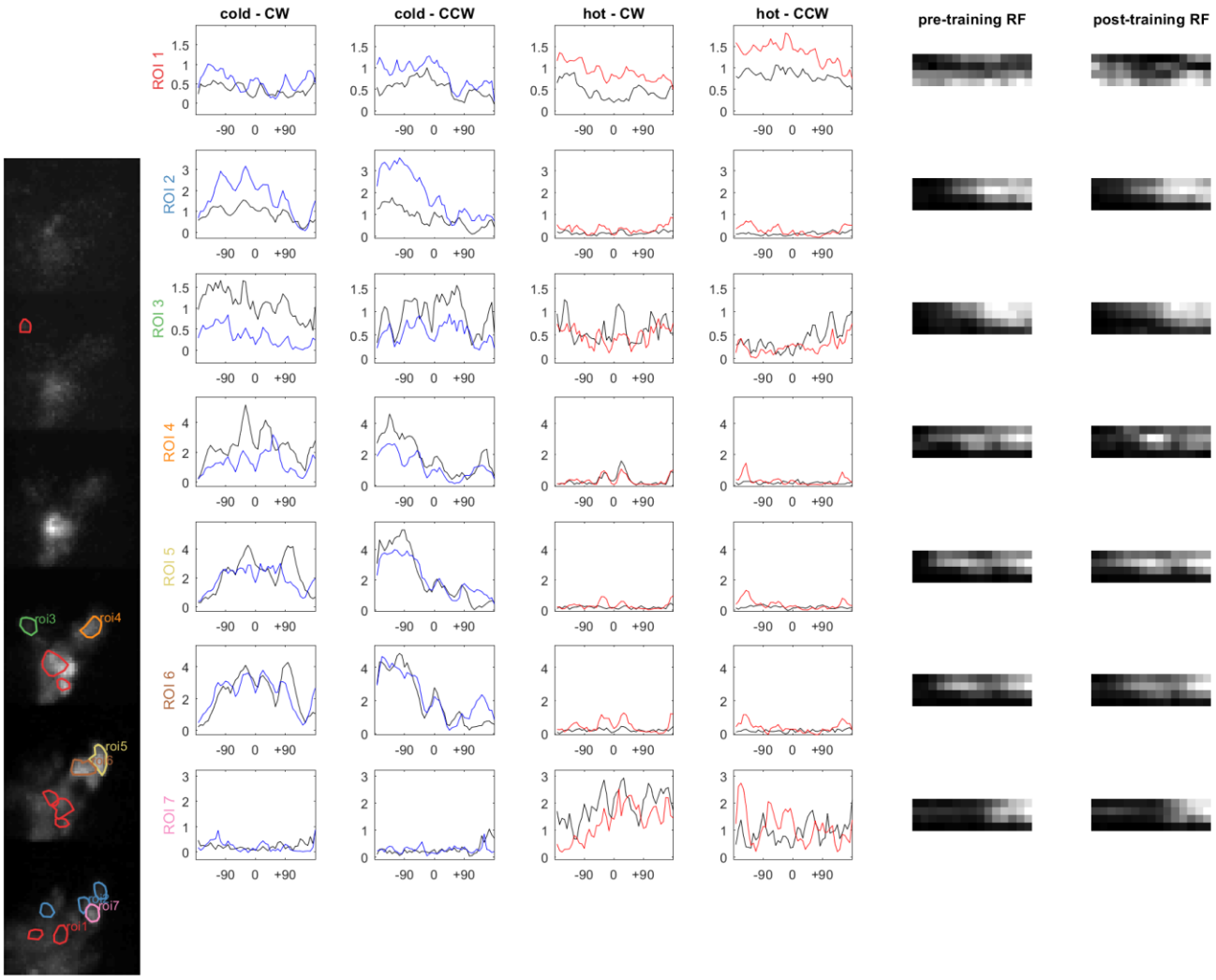
Wang, Y., Mamiya, A., Chiang, A-S., Zhong, Y (2008). Imaging of an early memory trace in the *Drosophila* mushroom body. *J. Neuroscience.* 28., 4368-4376.

Wustmann G, Rein K, Wolf R, Heisenberg M. (1996). A new paradigm for operant conditioning of Drosophila melanogaster. J Comp Physiol A; 179:429–436

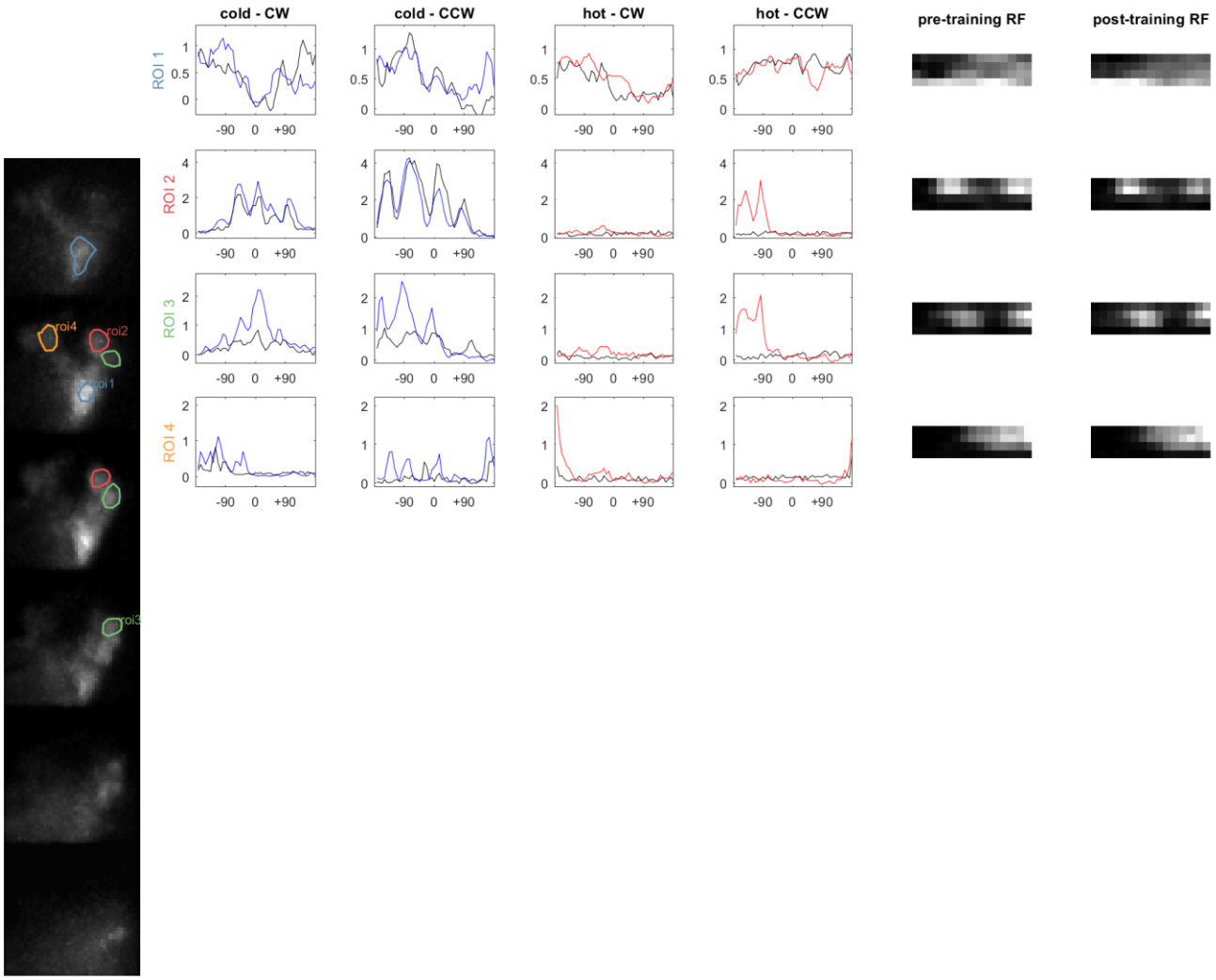
Xu., S.Y., Cang, C.L., Liu, X.F., Peng, Y.Q., Ye, Y.Z, Ye, Z., Zhao, Q., Guo, A.K. (2006). Thermal nociception in adult *Drosophila*: behavioral characterization and the role of the *painless* gene. *Genes, Brain, and Behavior.* 5, 602-613.

Appendix

Position A Fly 1



Position A Fly 2



Position A Fly 3 – 1 of 2

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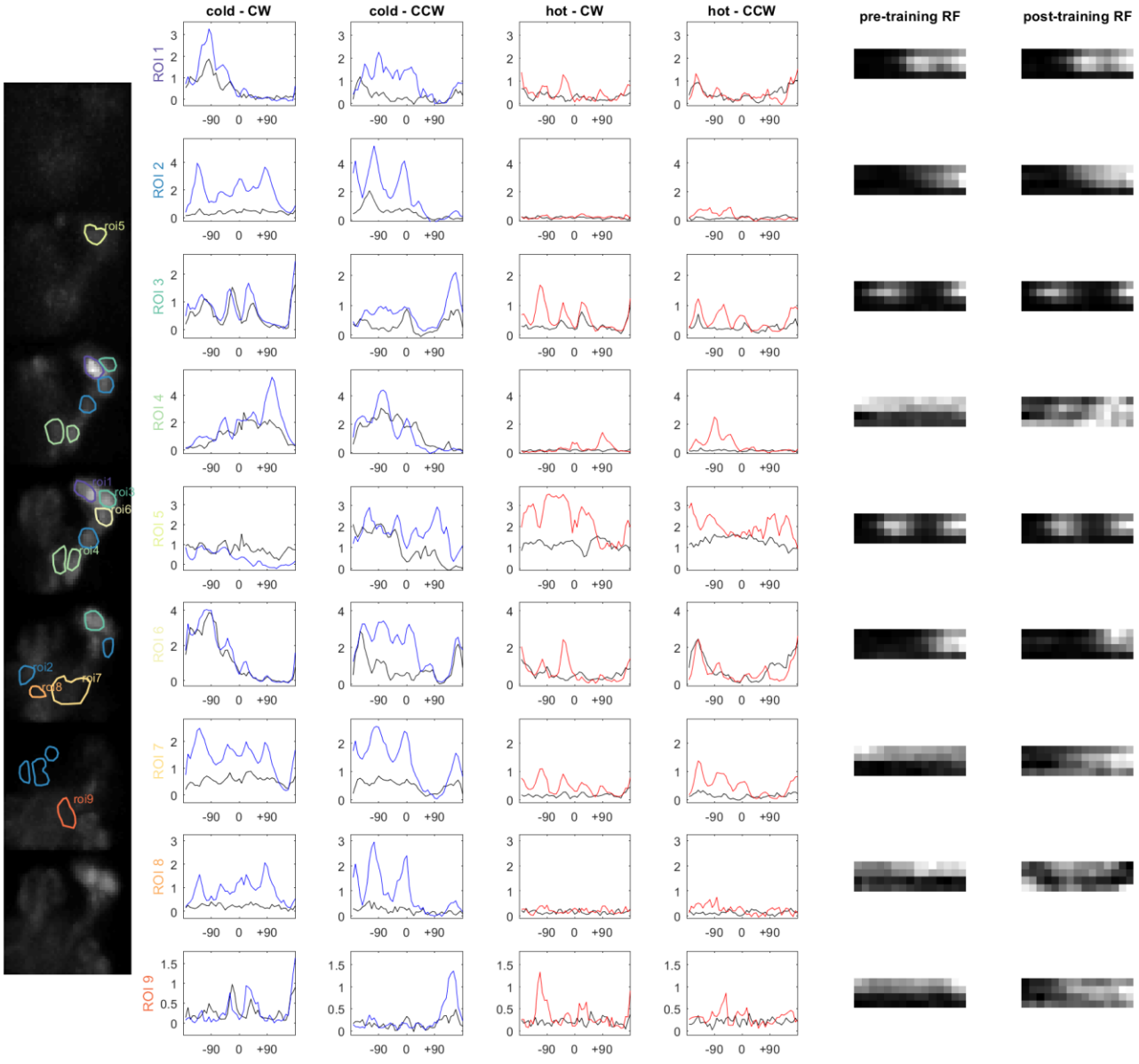
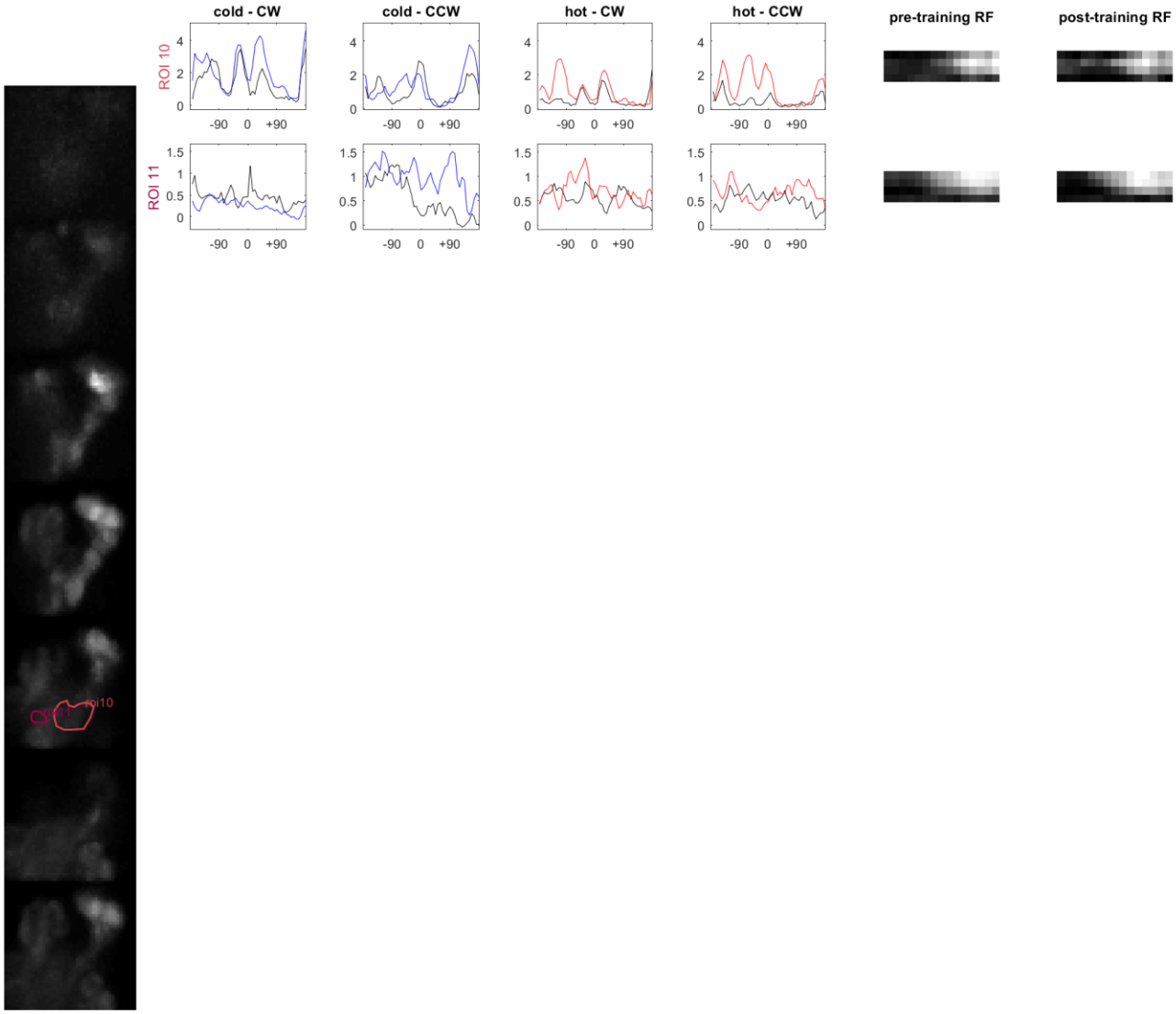


Position A Fly 3 – 2 of 2



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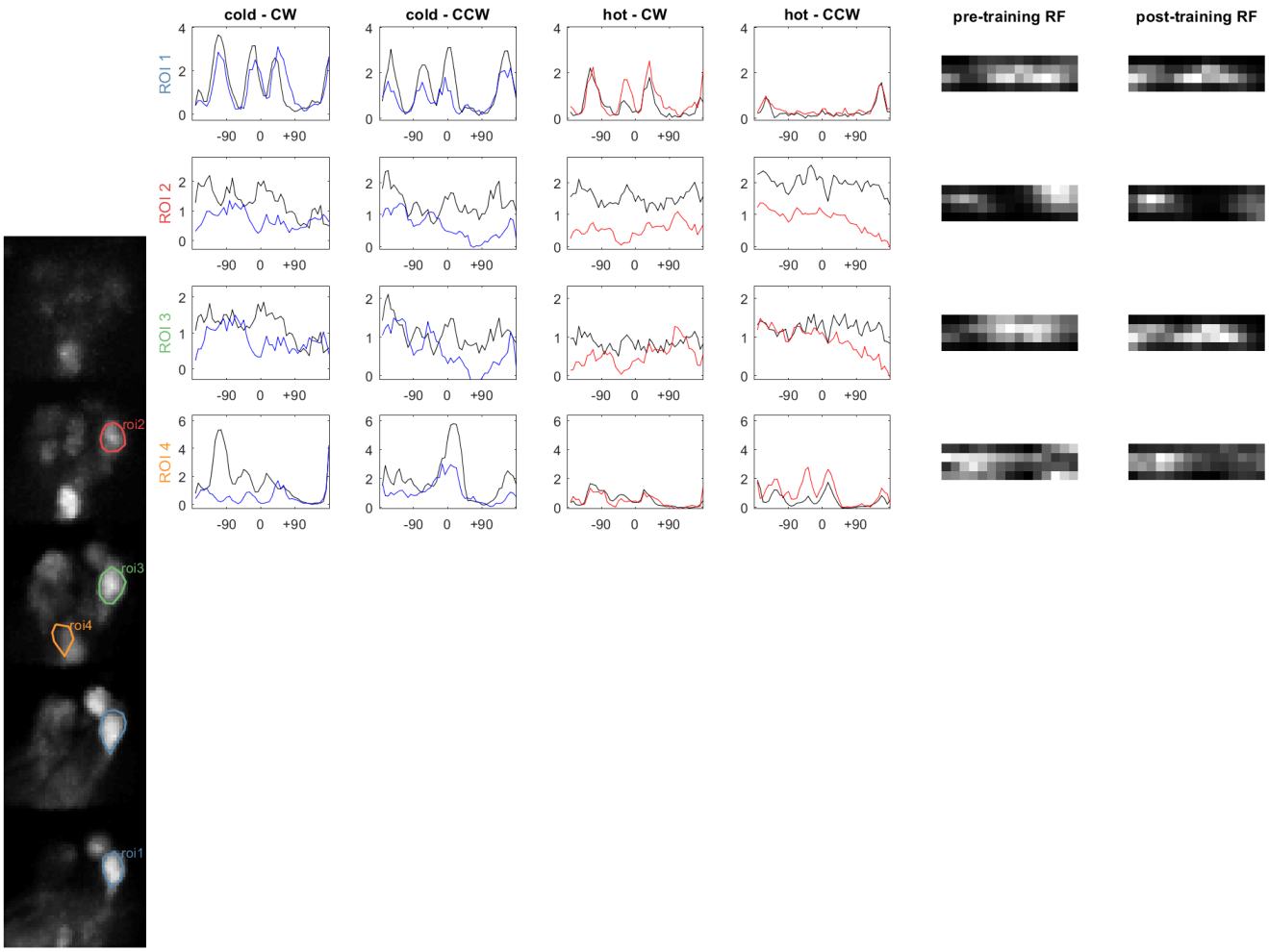

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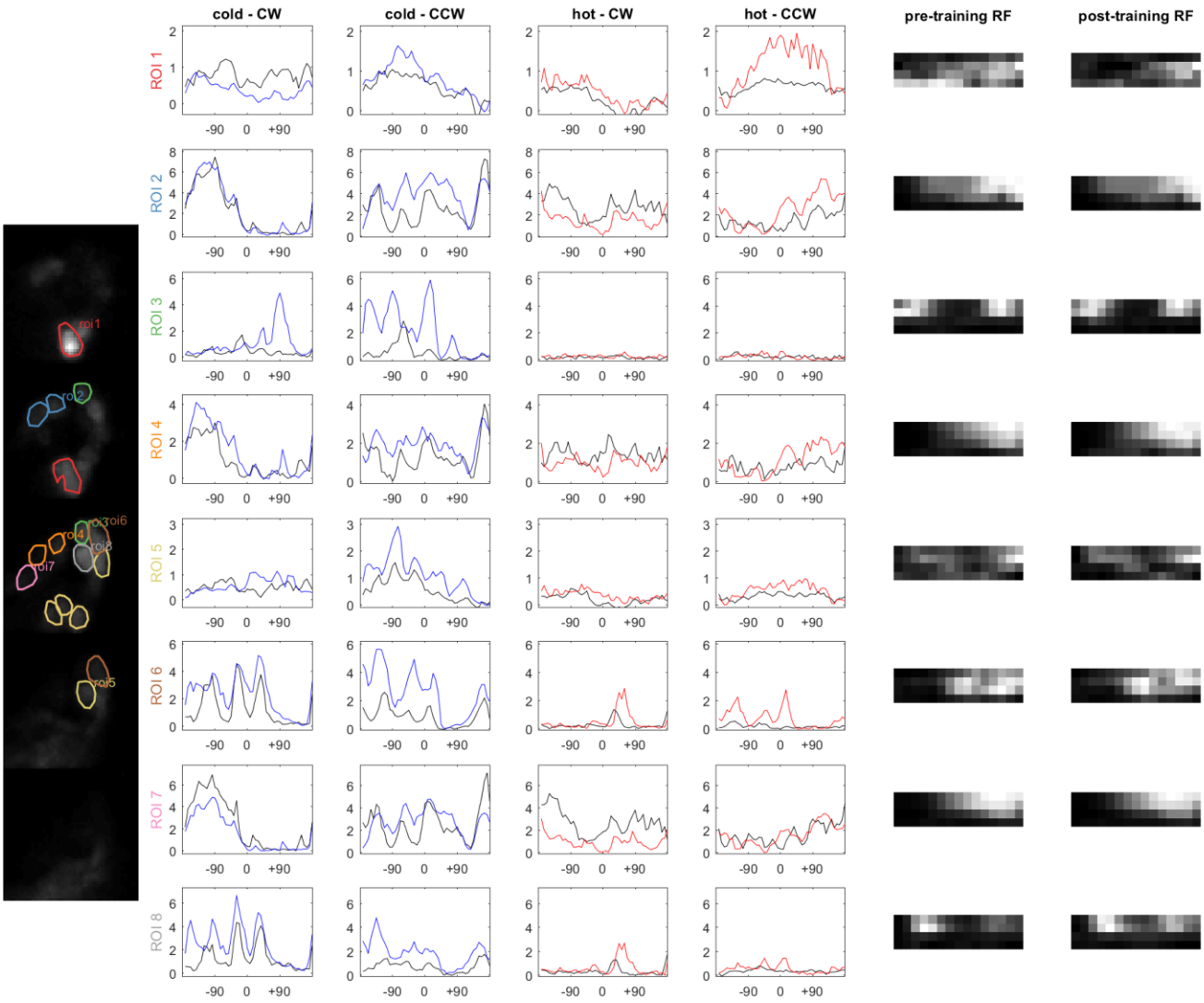
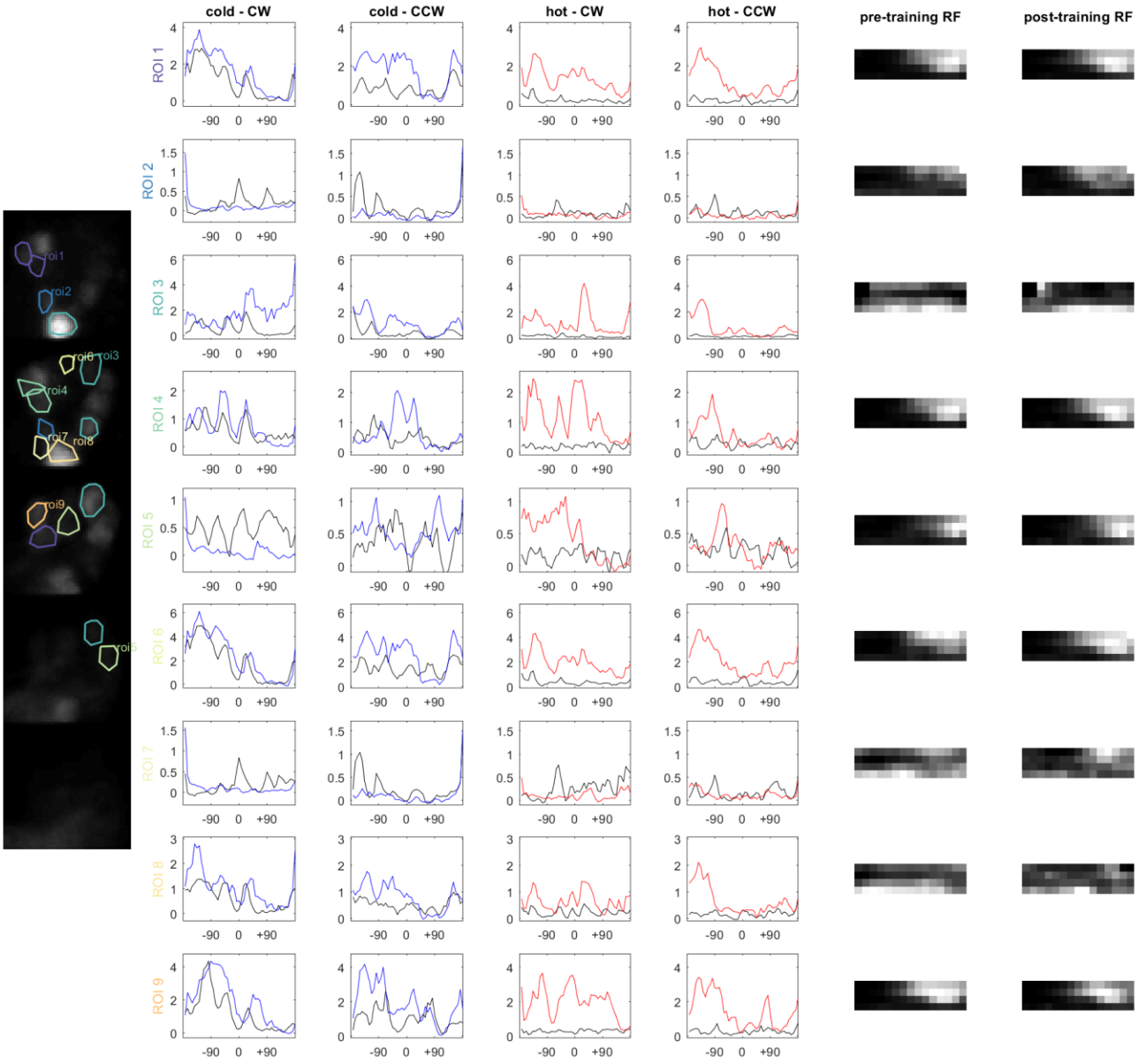


Position A Fly 6 – 1 of 2



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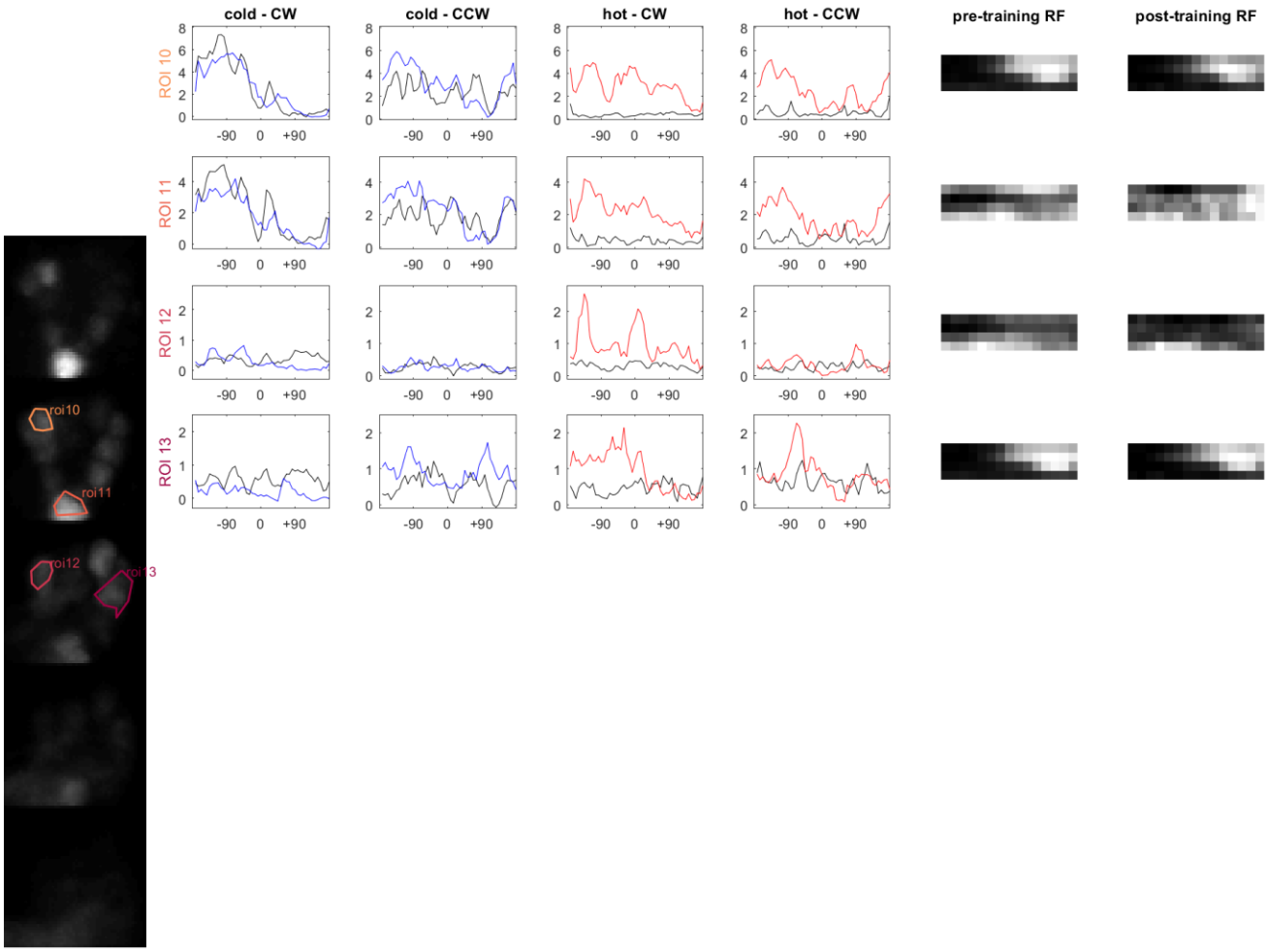
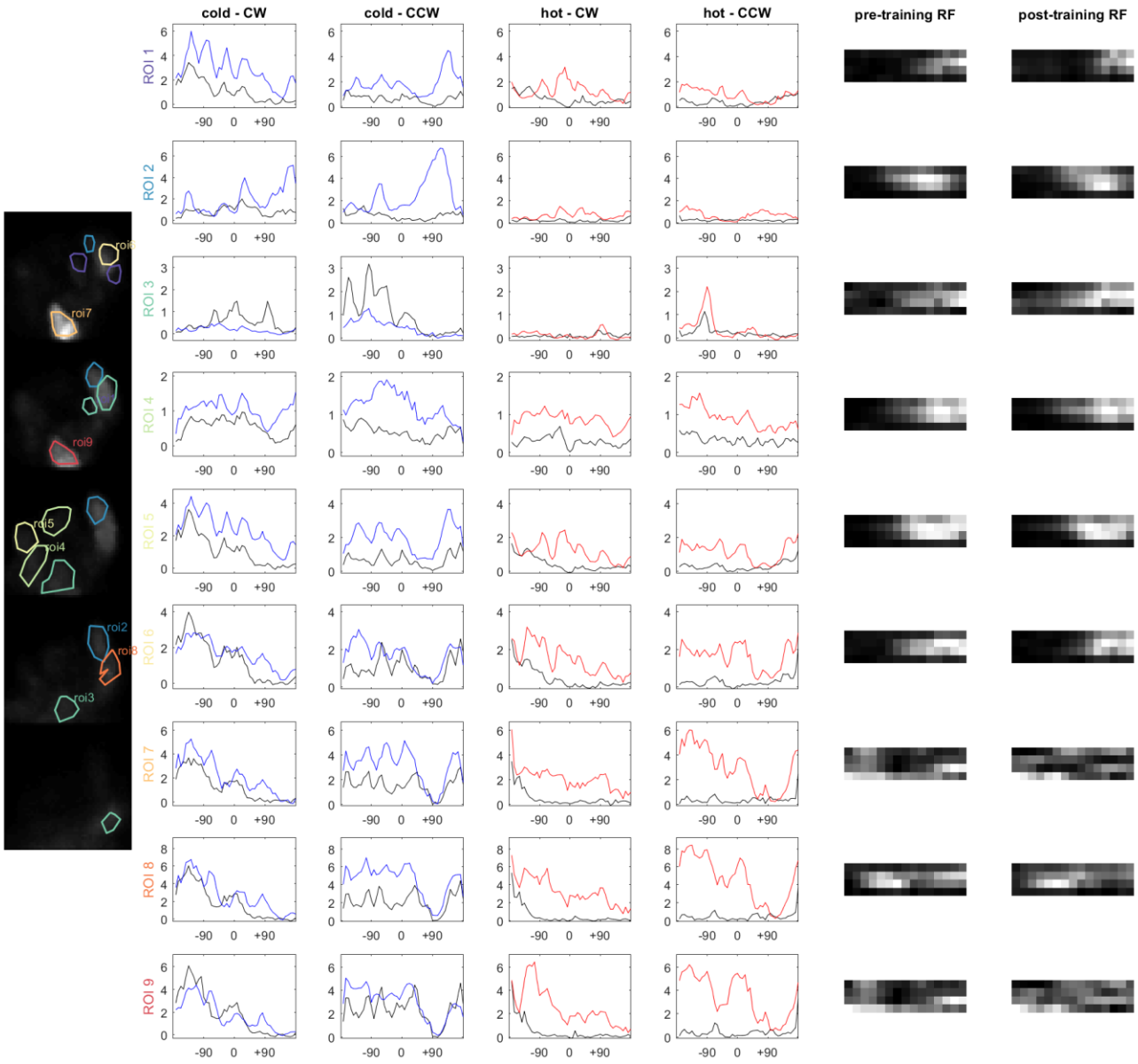


Position A Fly 7 – 1 of 2



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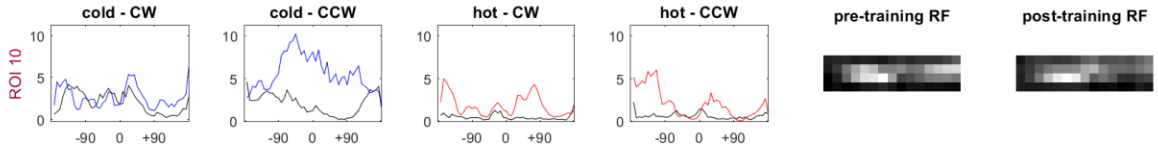
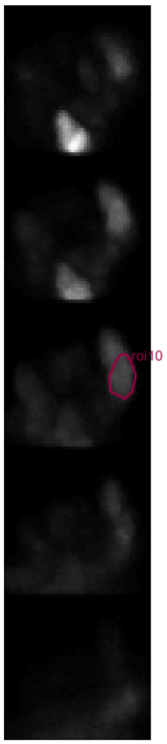
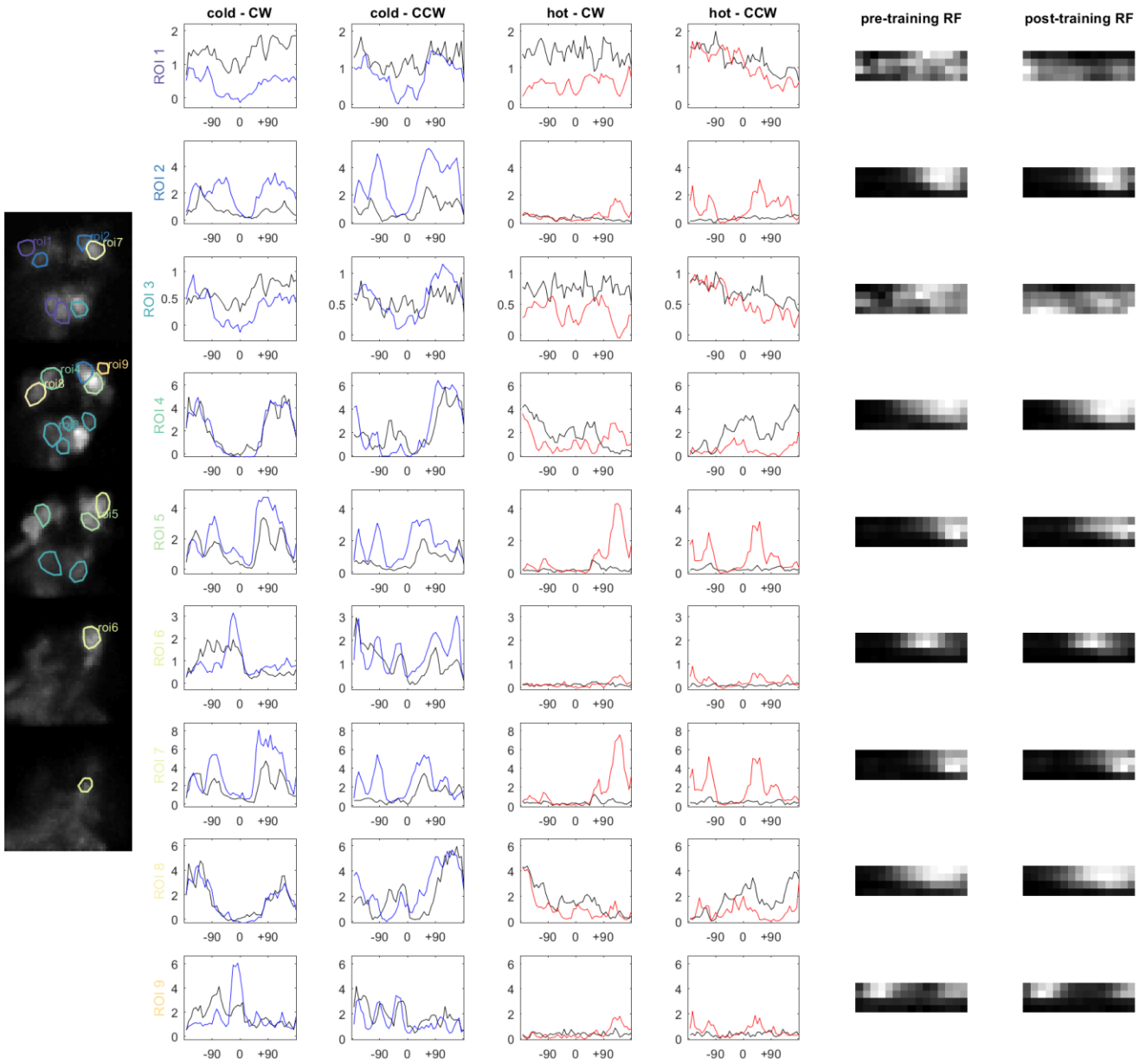
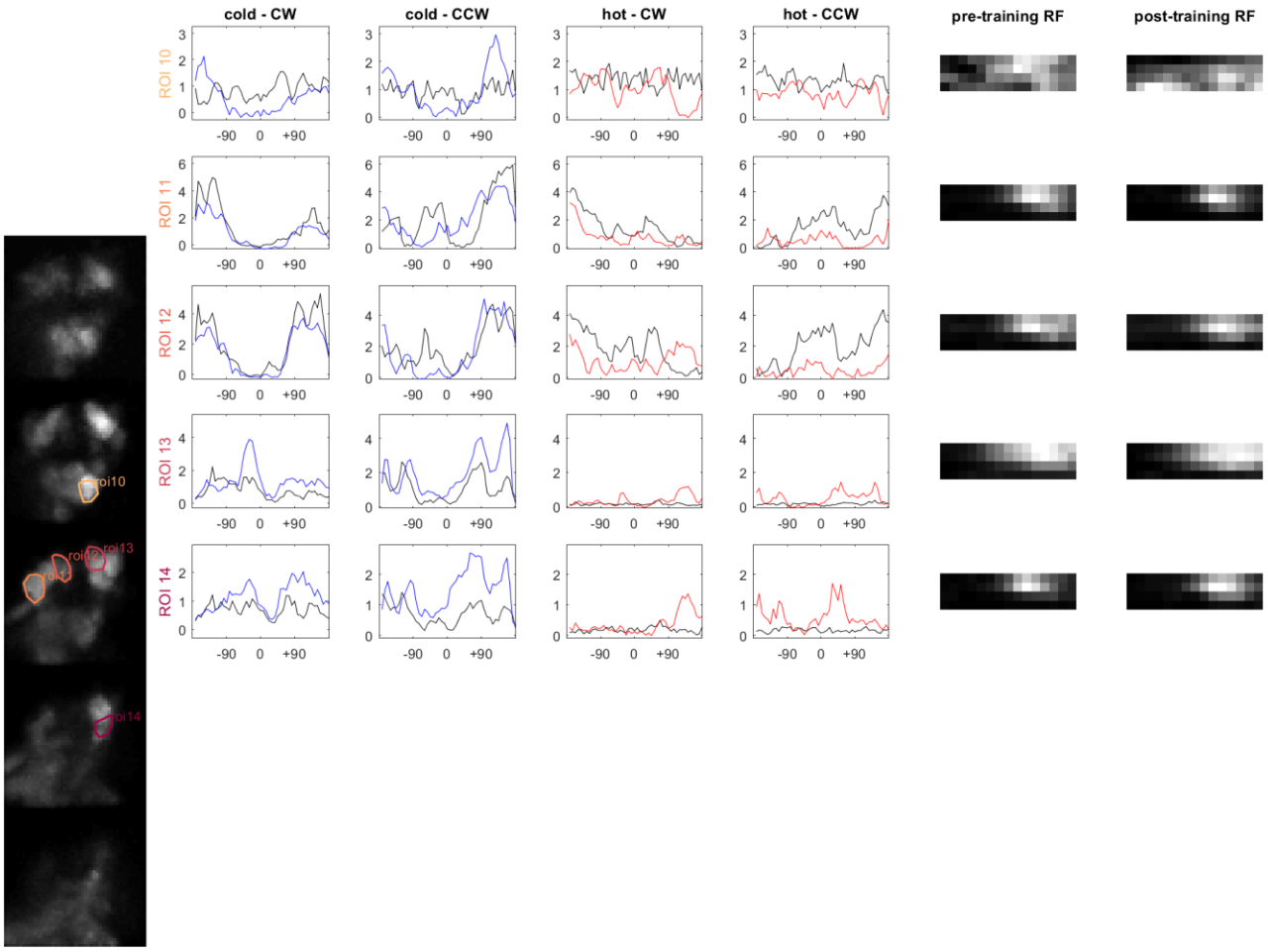




Position B Fly 1 – 1 of 2



Position B Fly 1 – 2 of 2



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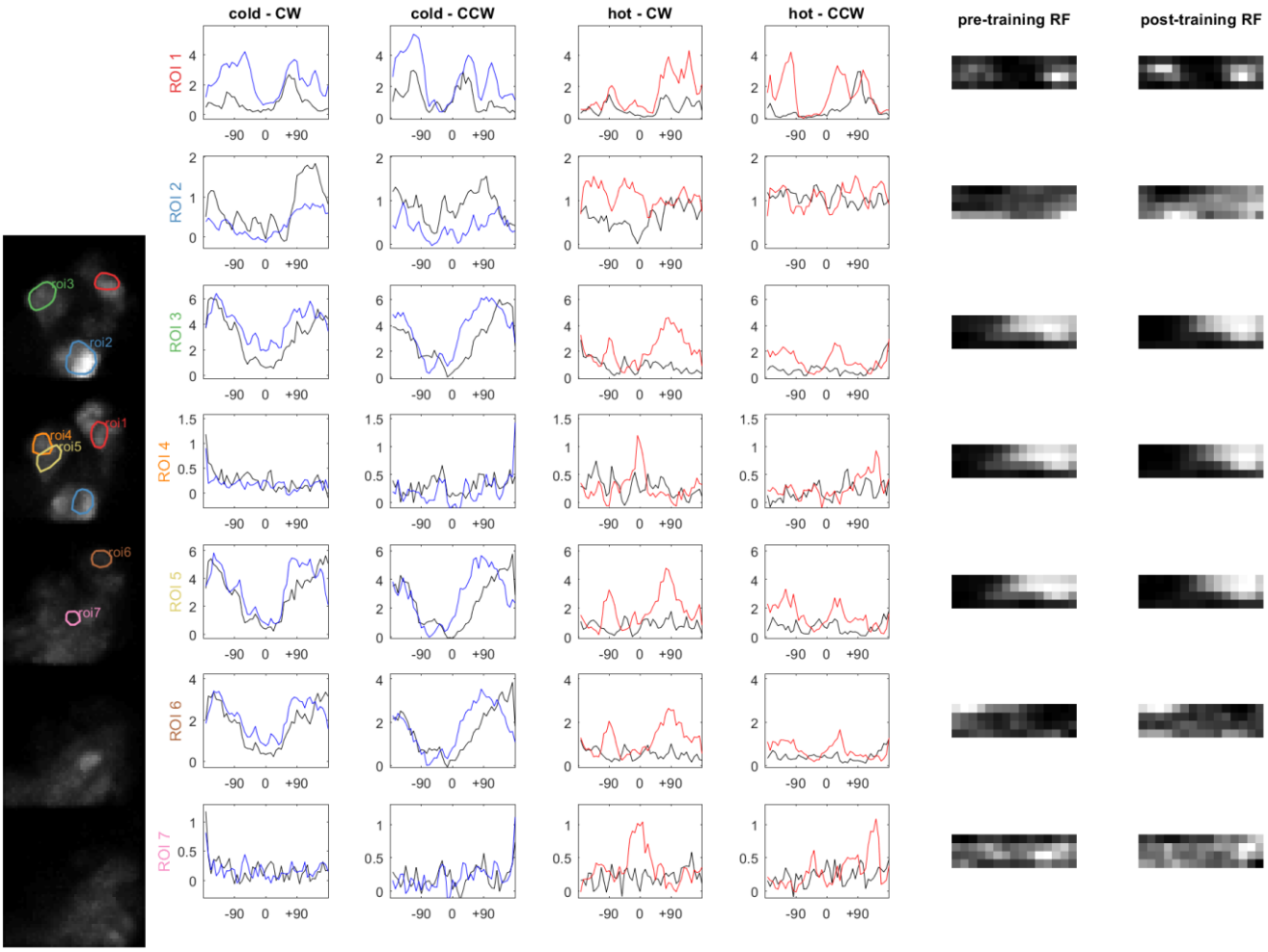
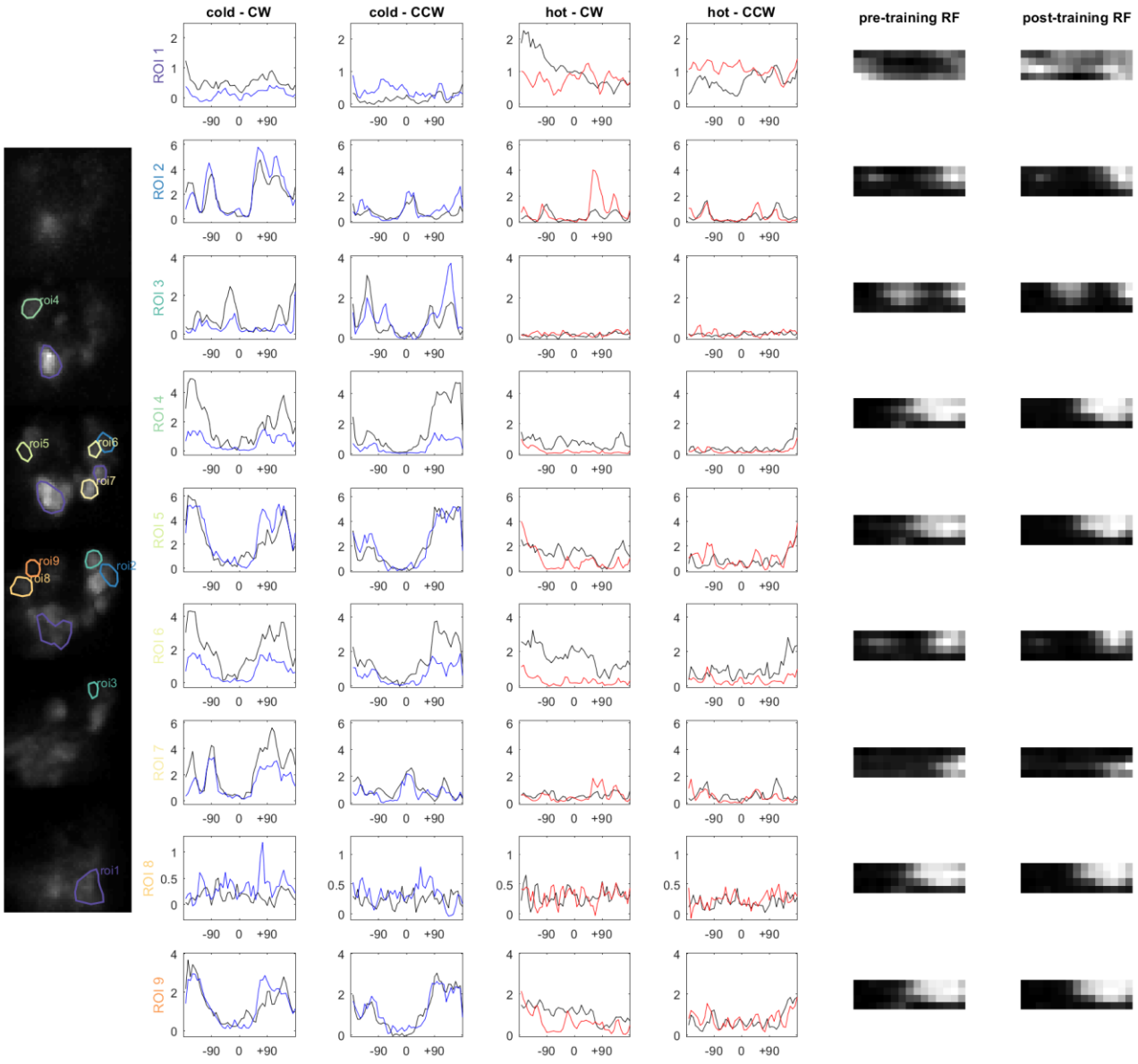
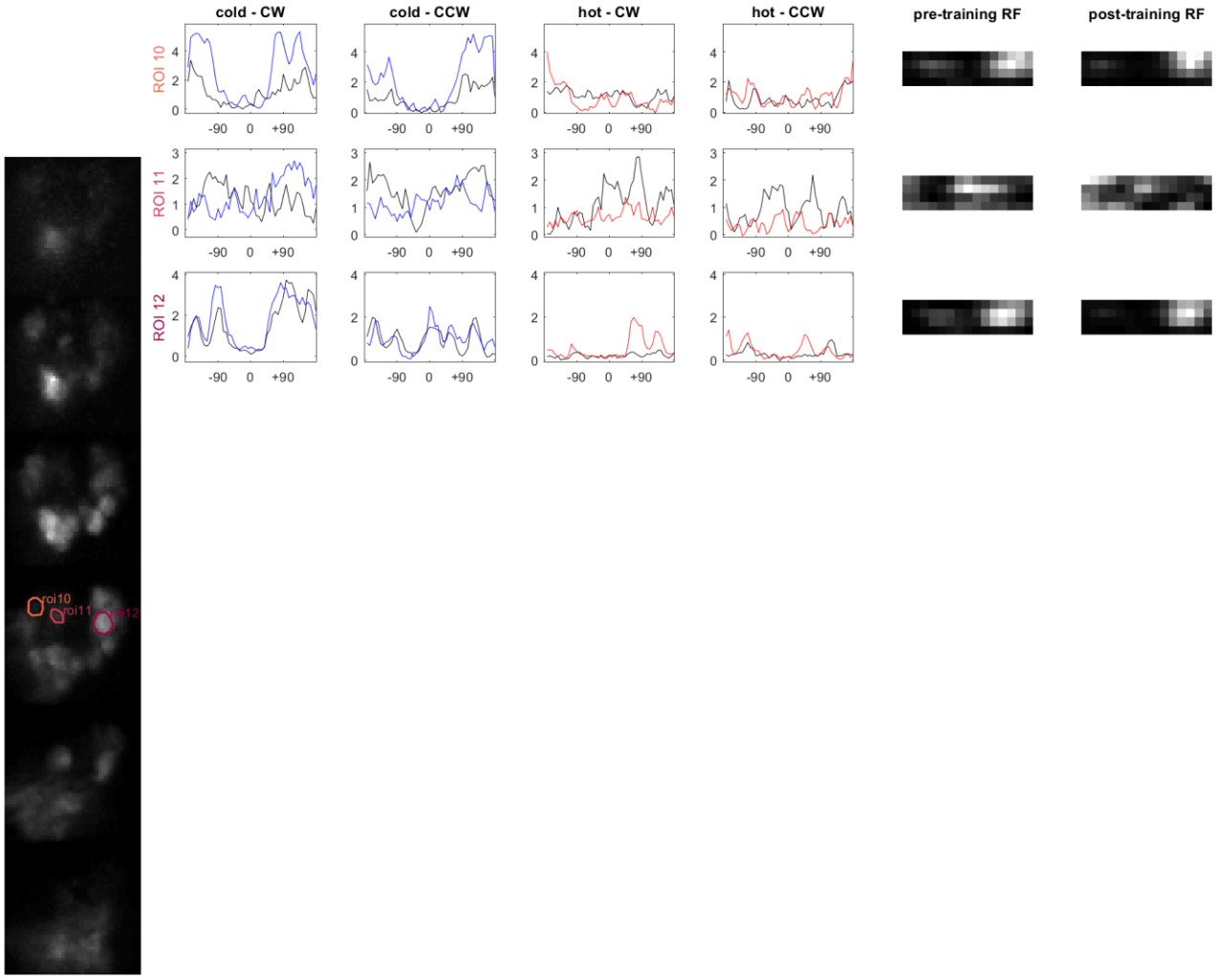


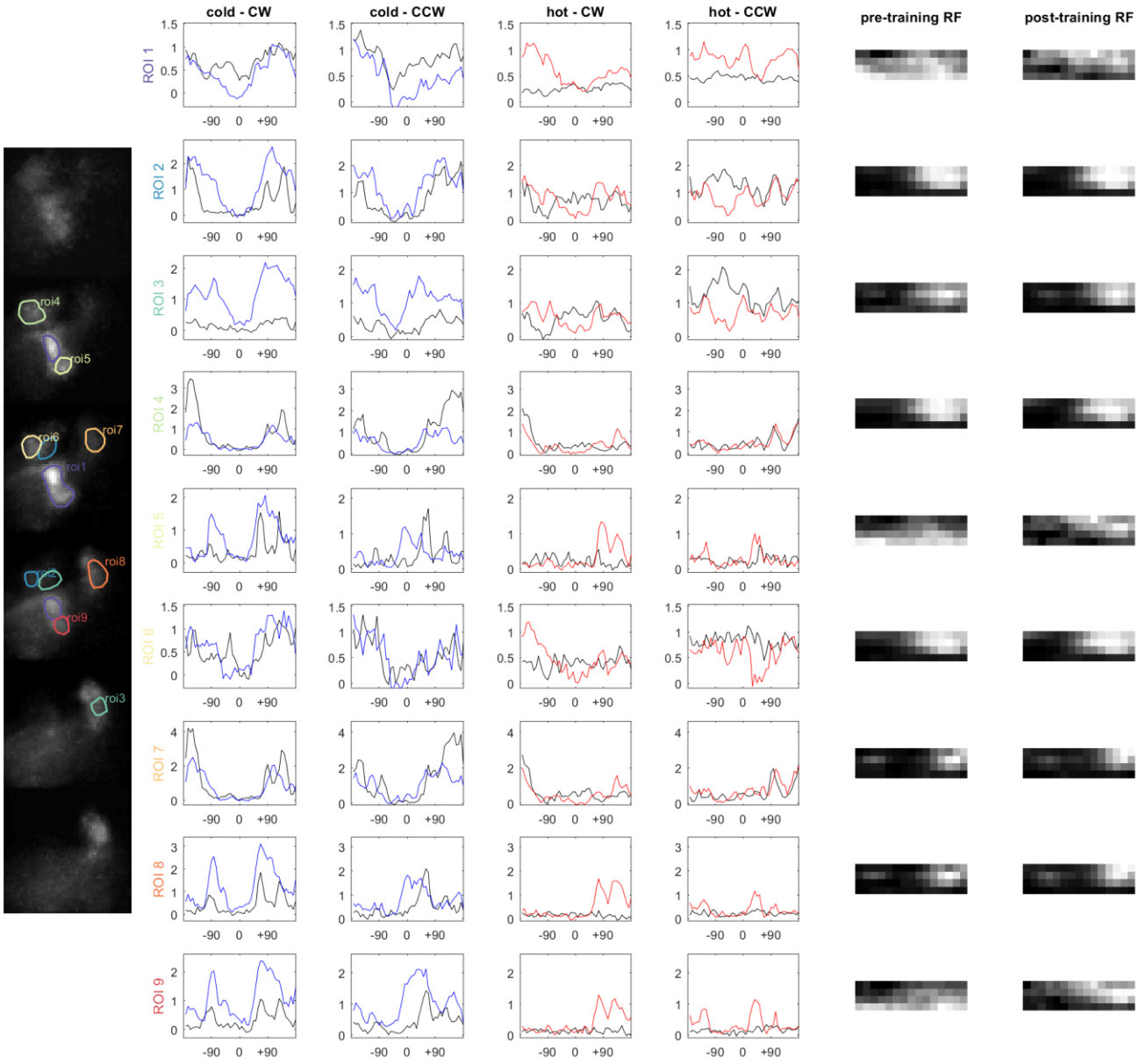
Position B Fly 3 – 1 of 2



Position B Fly 3 – 2 of 2



Position B Fly 4 – 1 of 2



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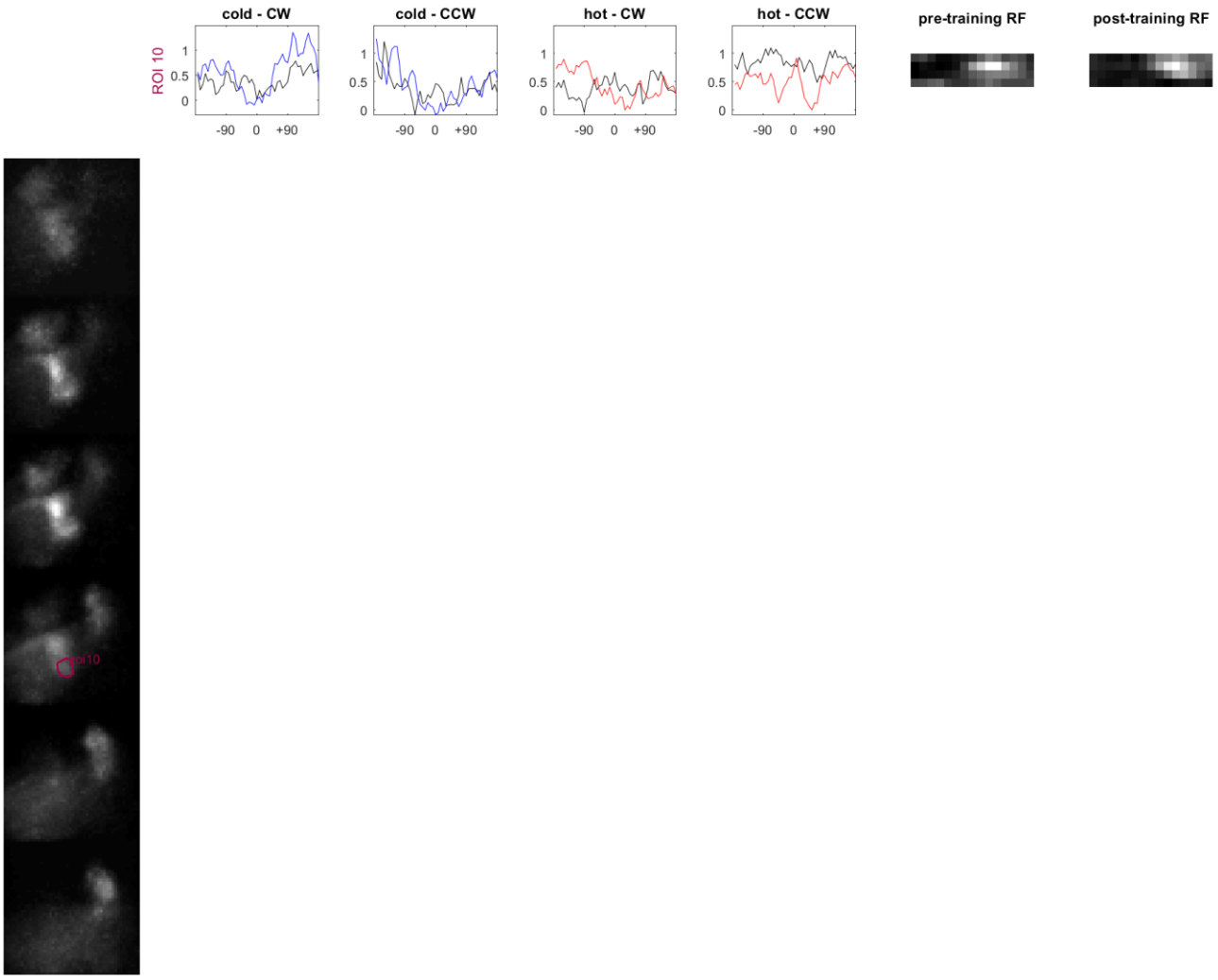

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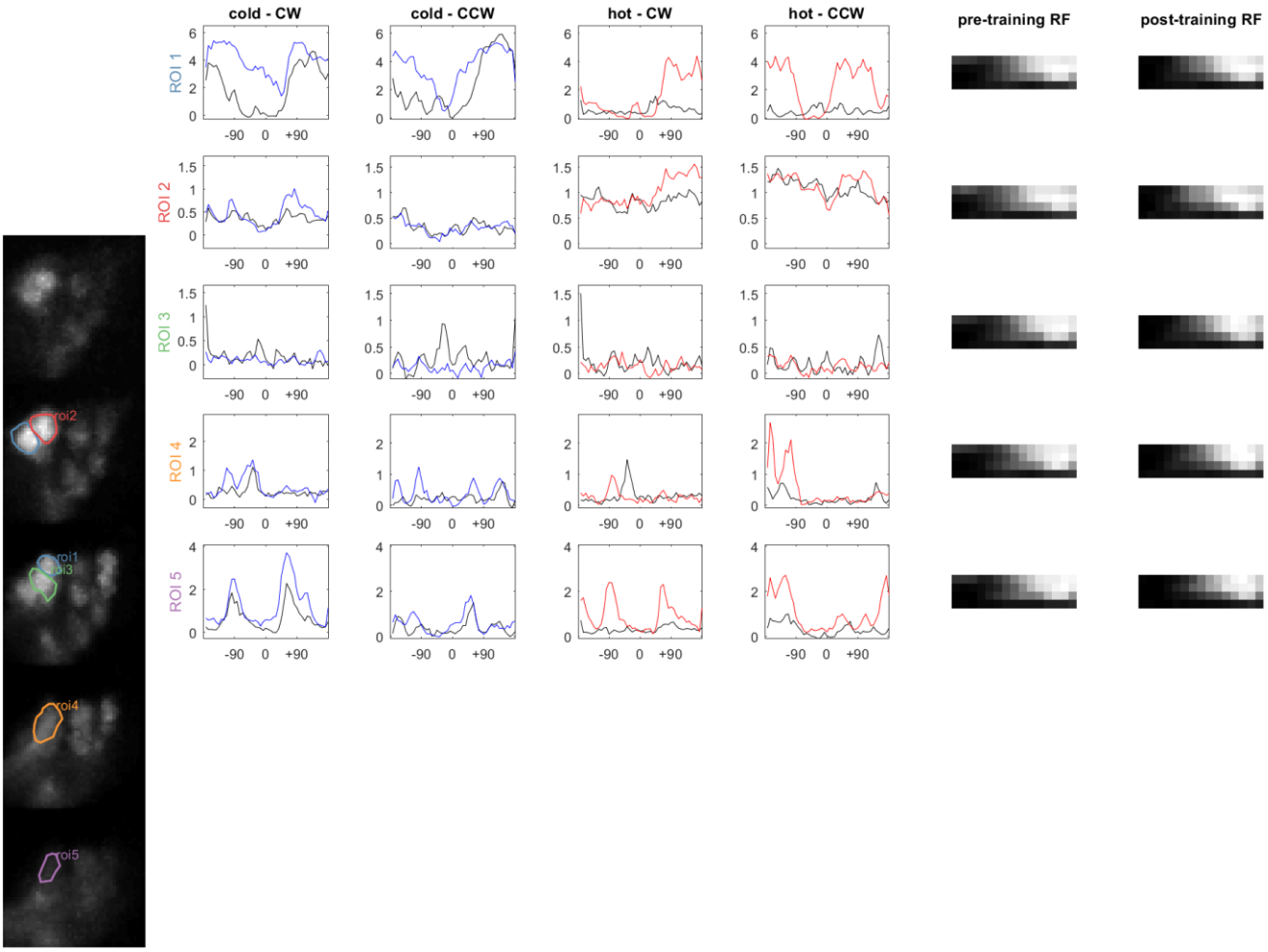

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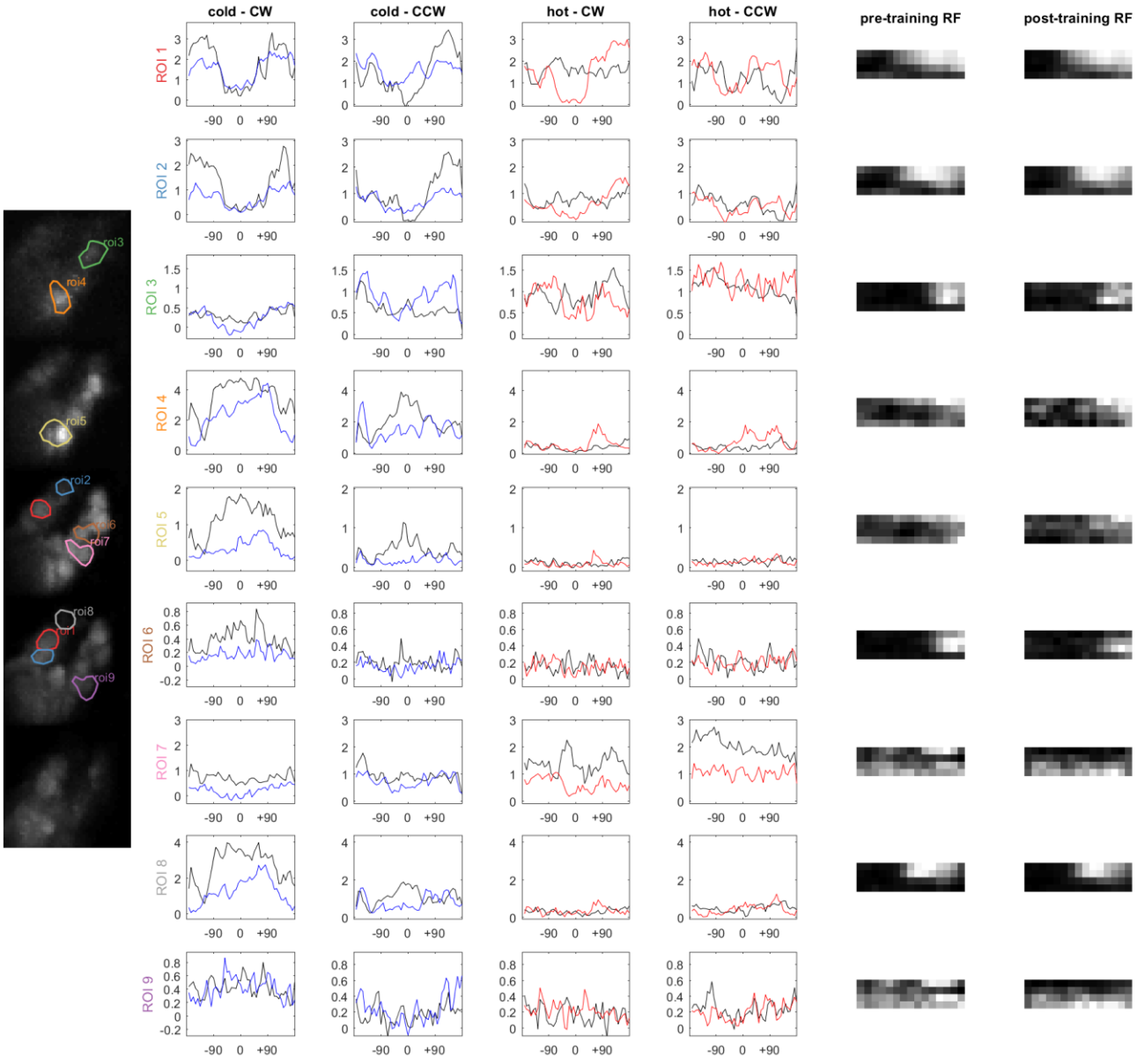
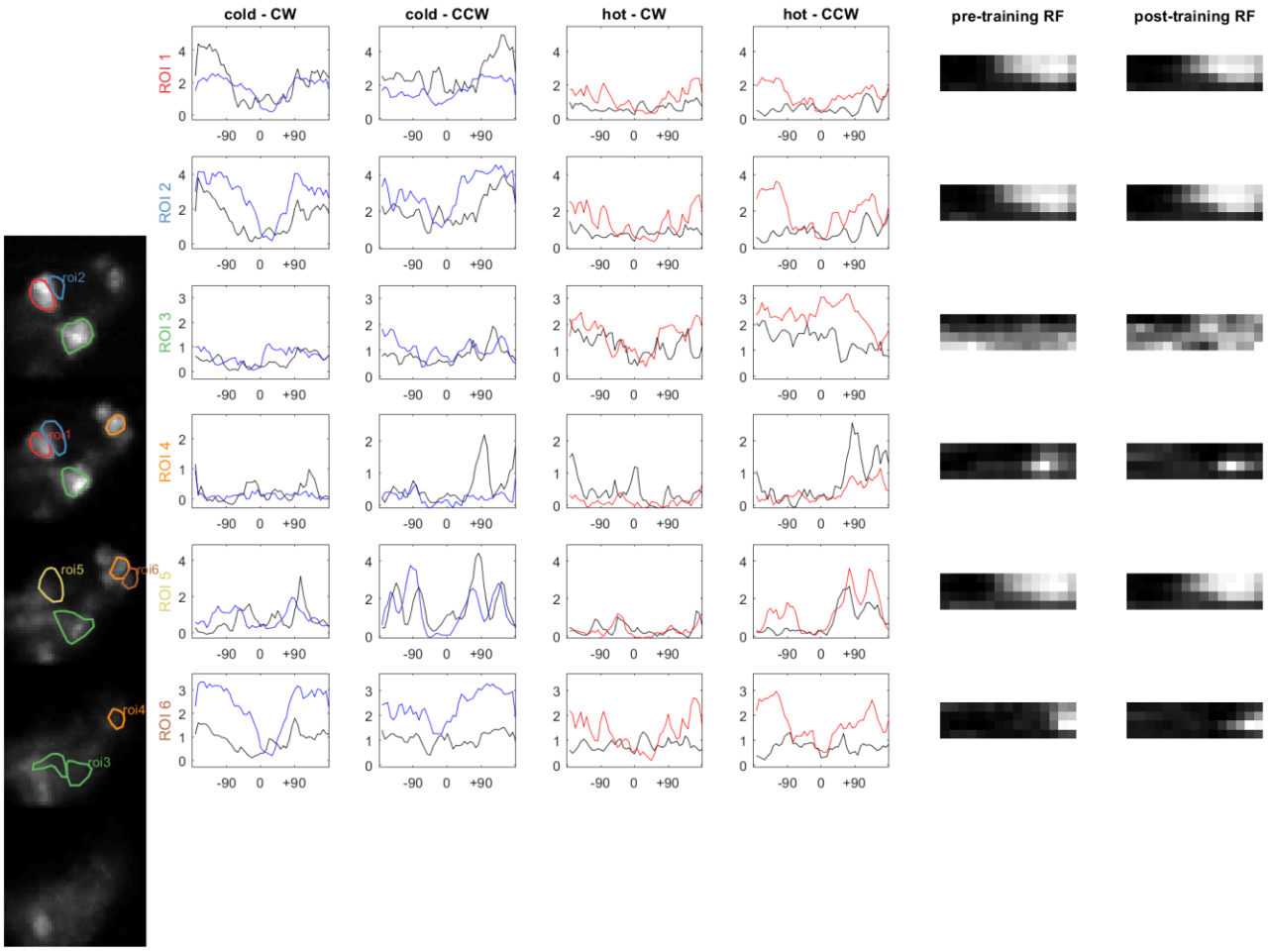
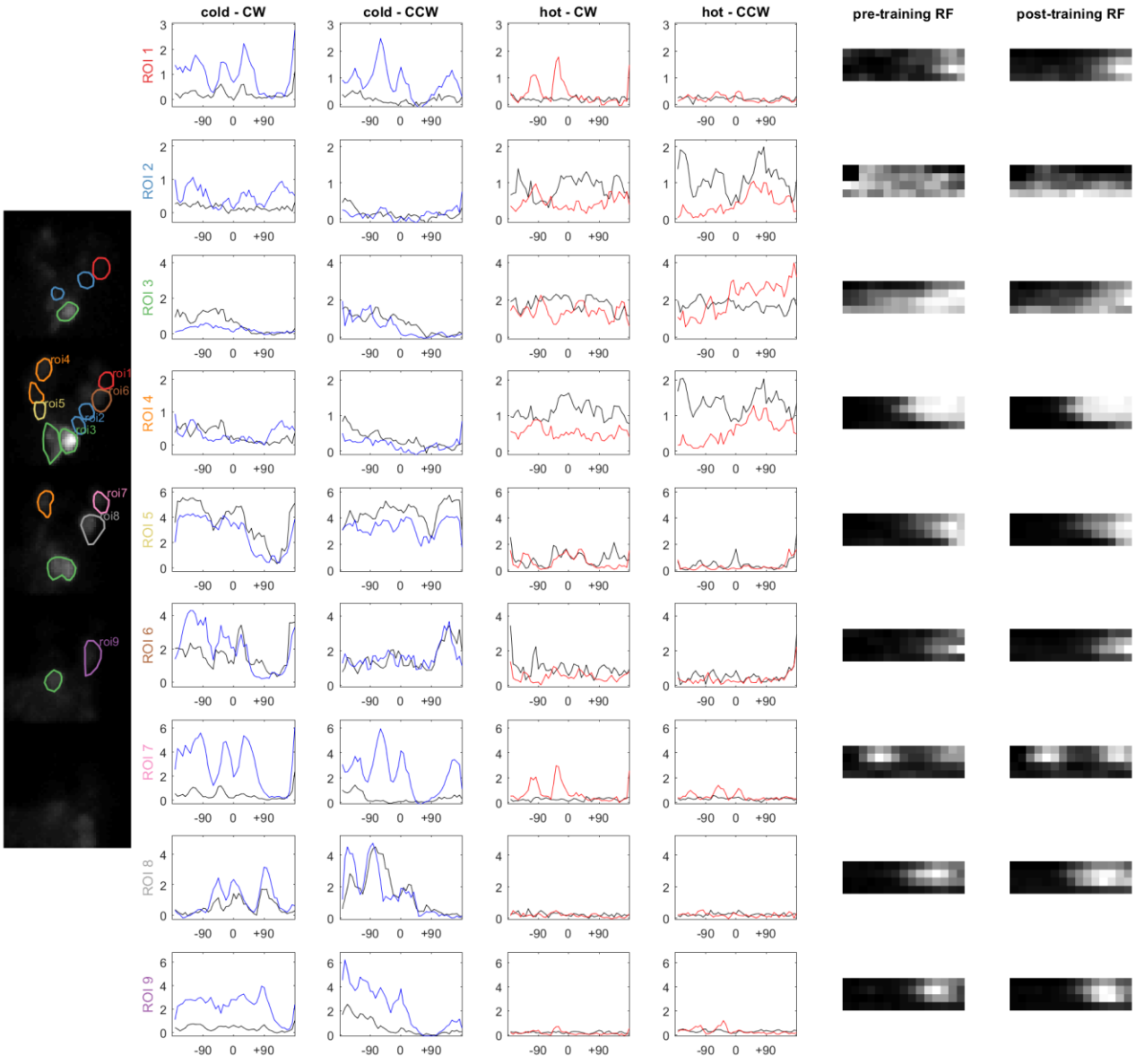


Position B Fly 7



Control Fly 1



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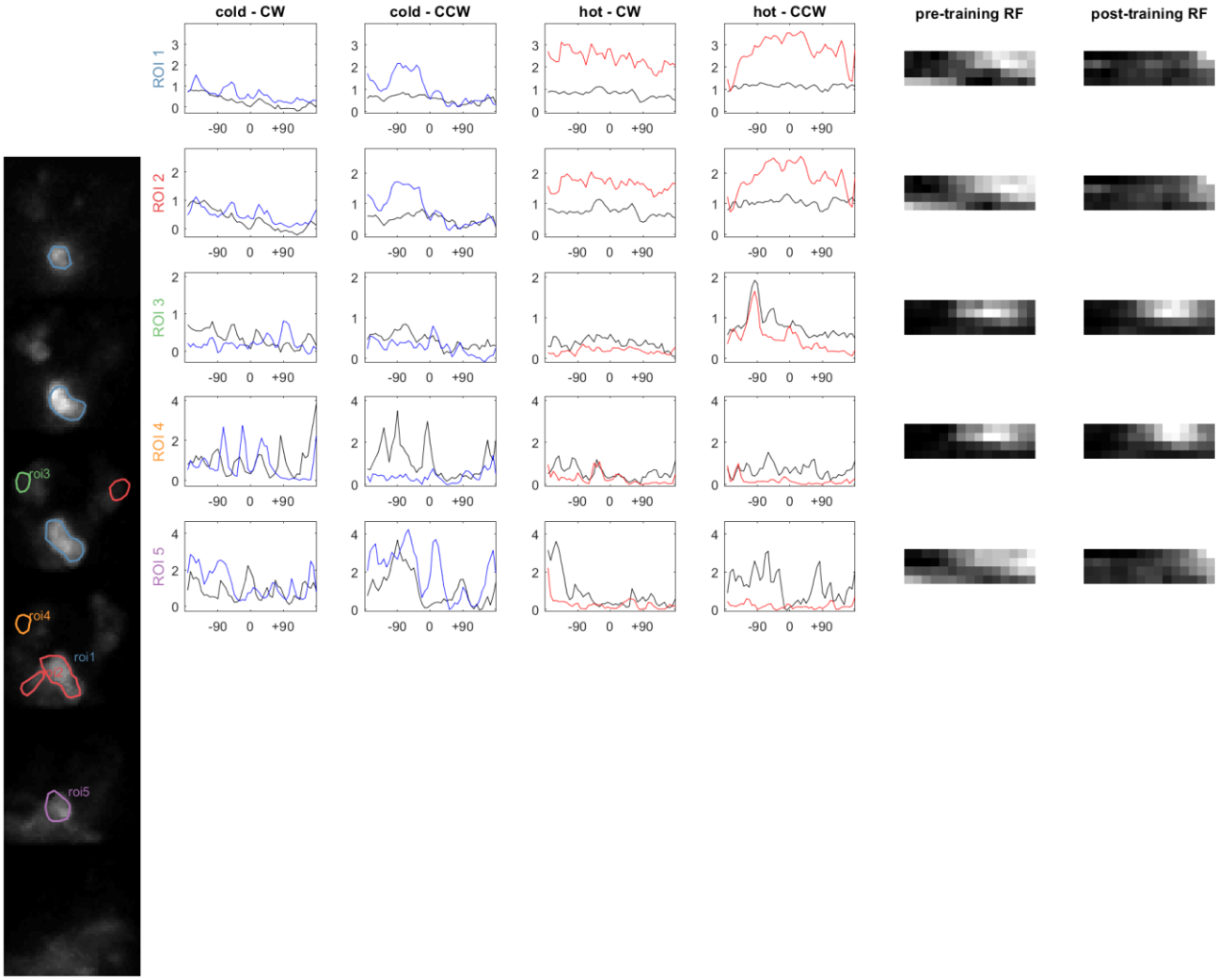

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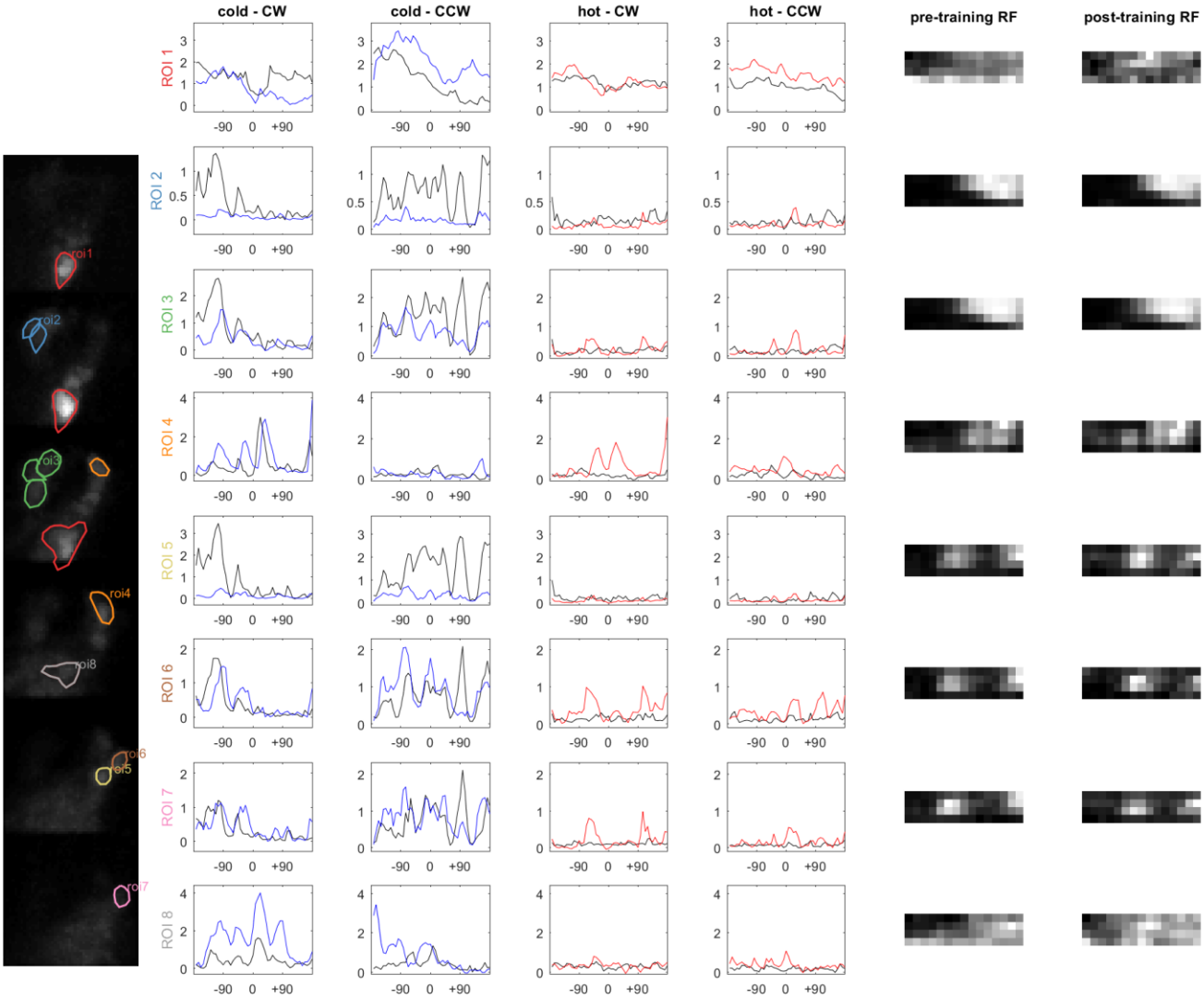

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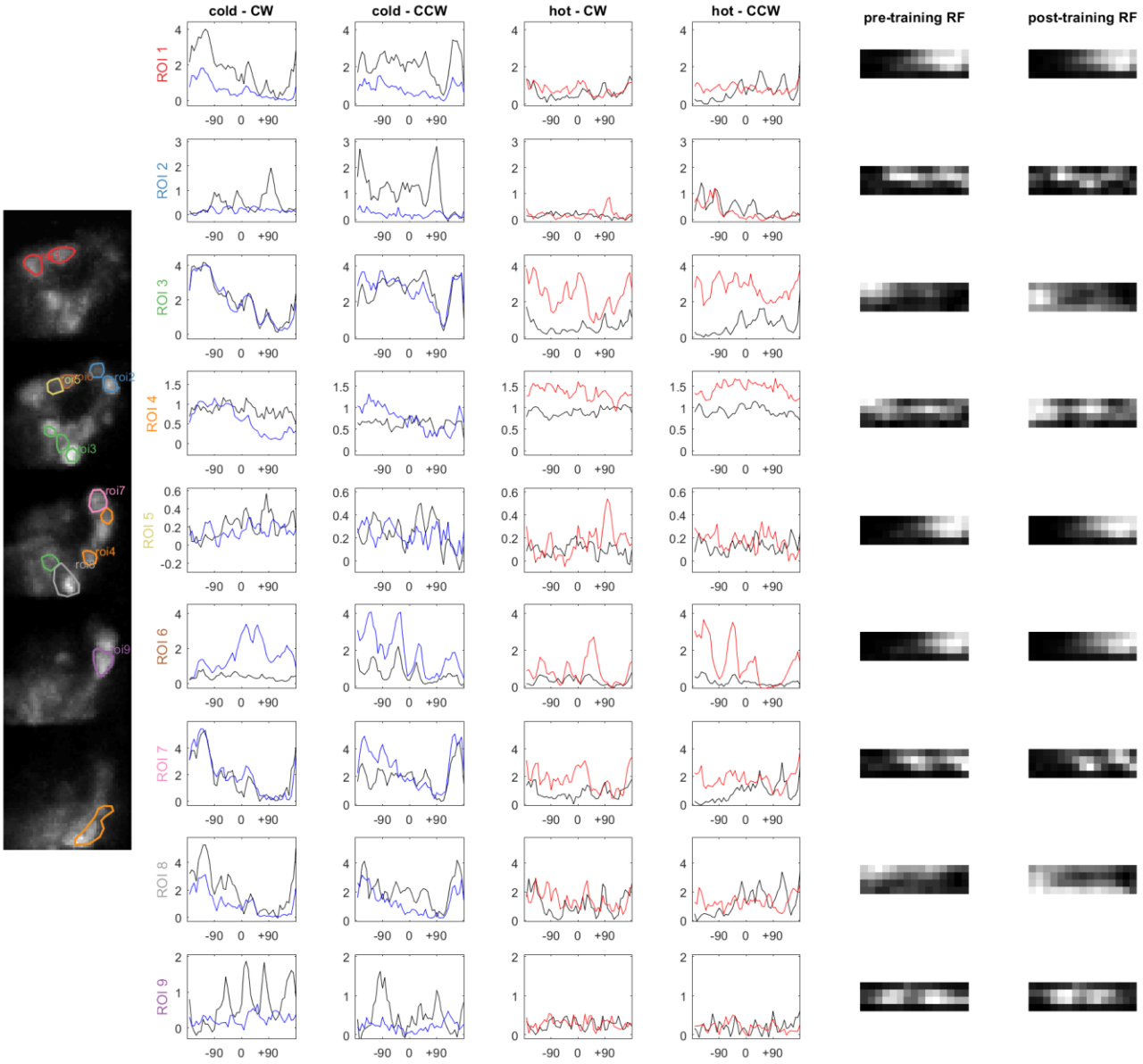
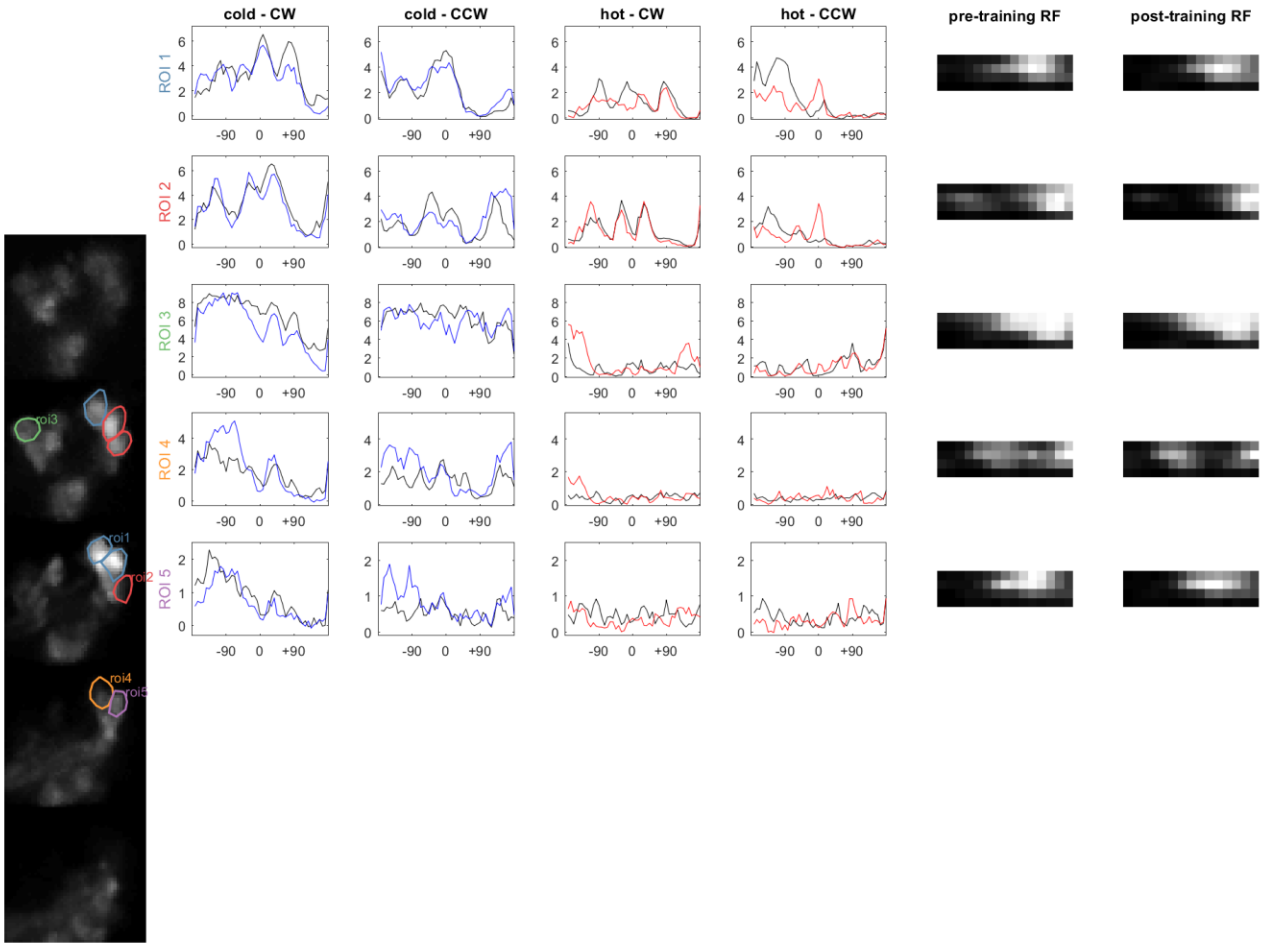
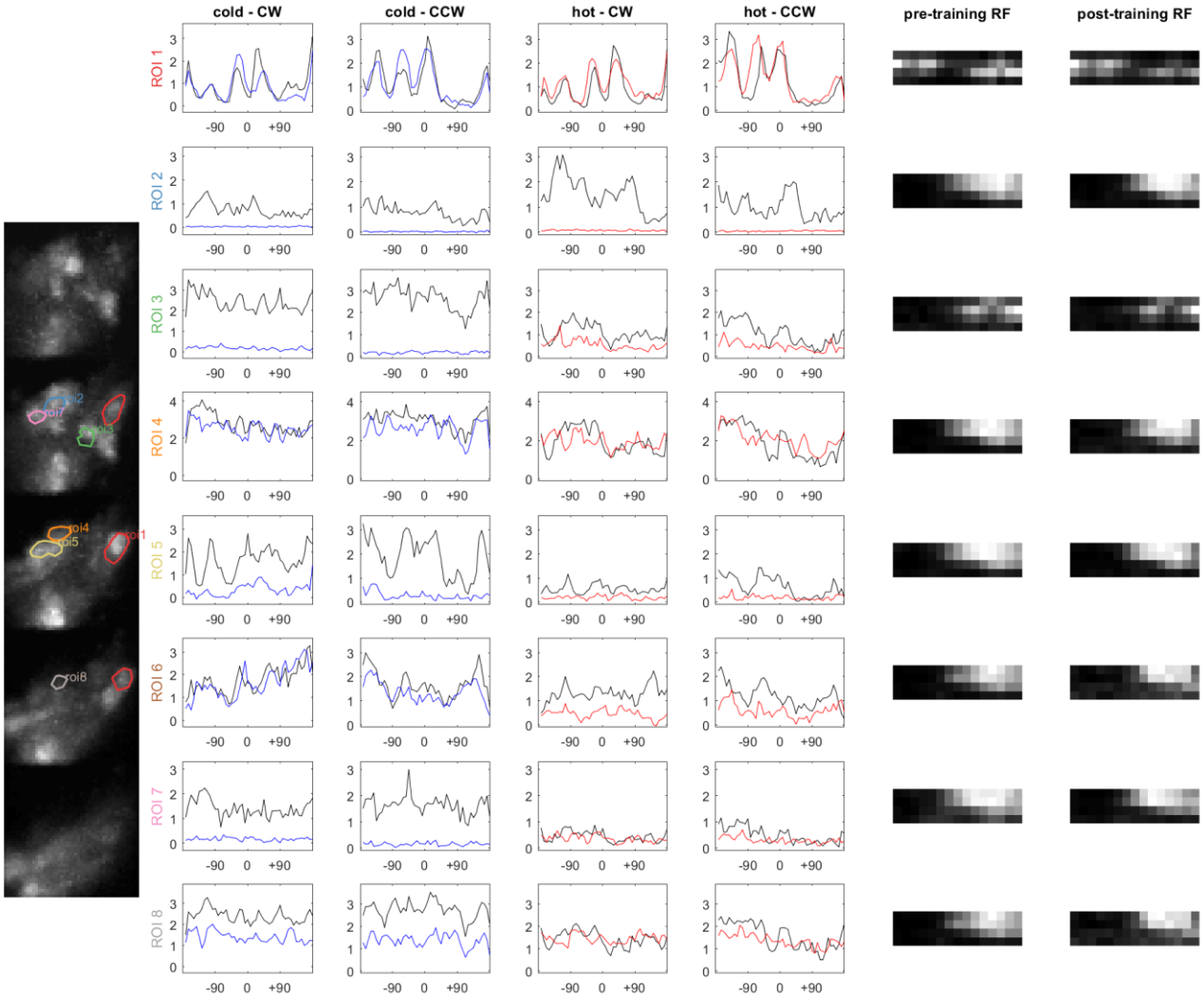


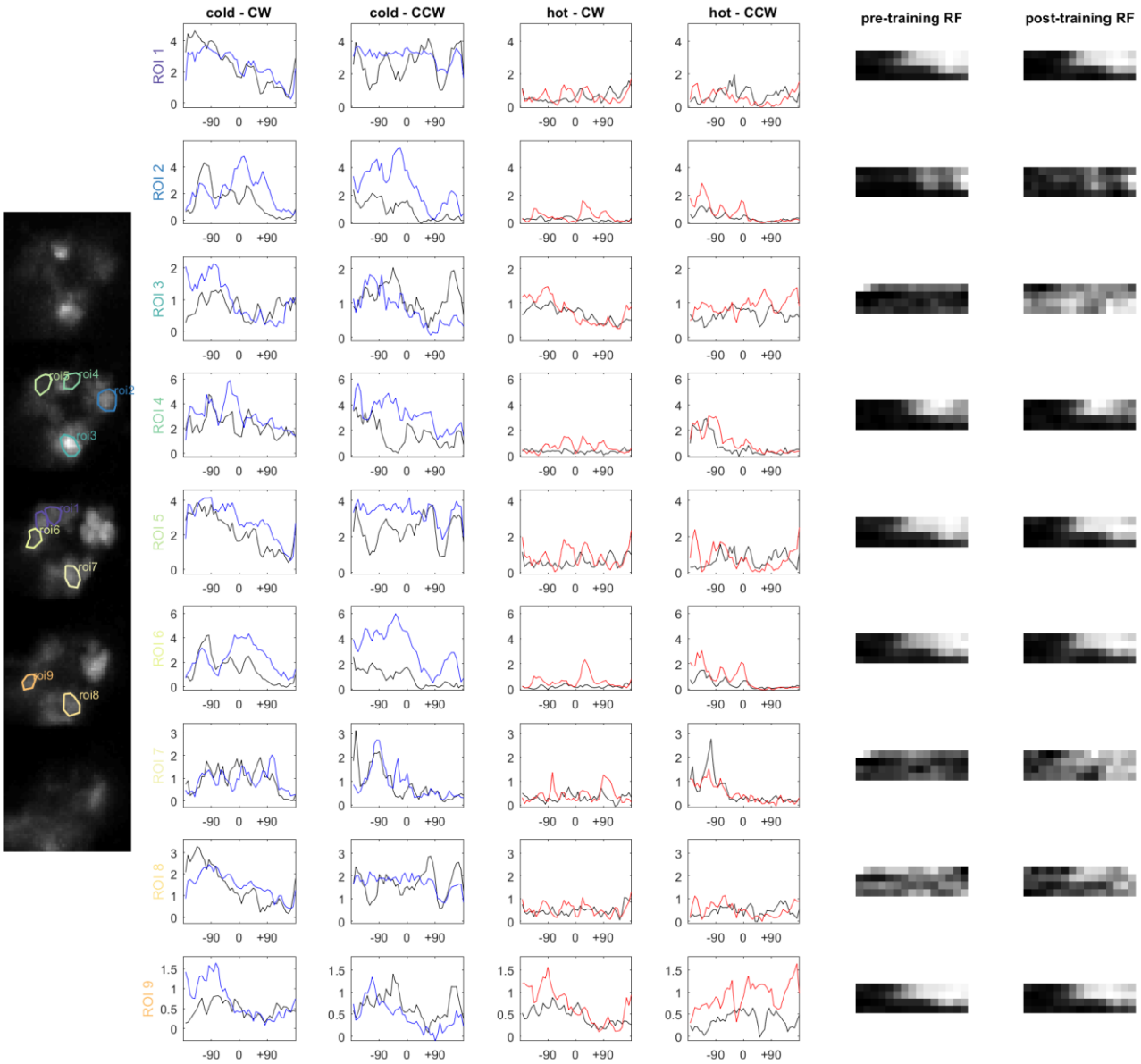
Control Fly 5



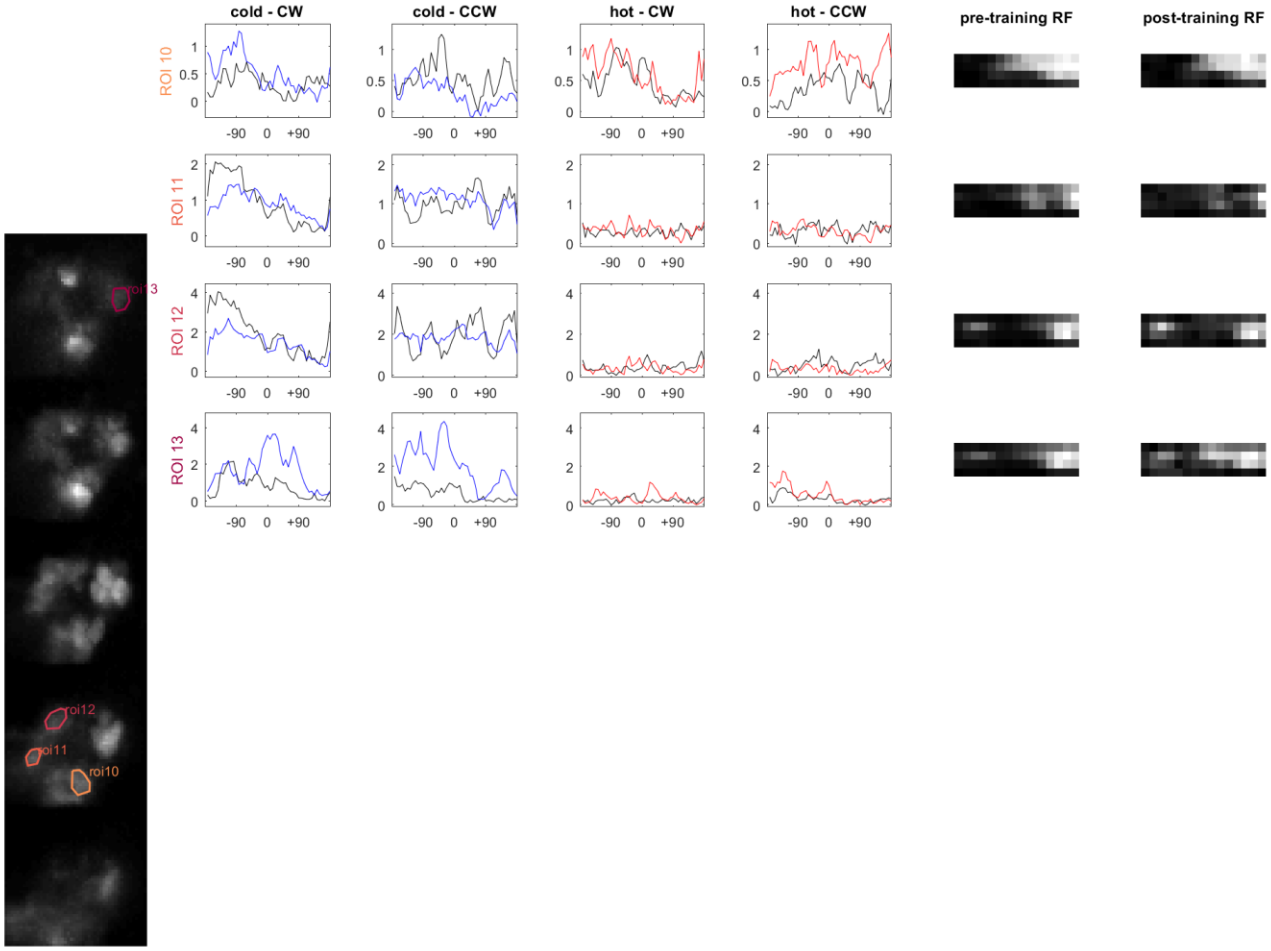
Control Fly 6



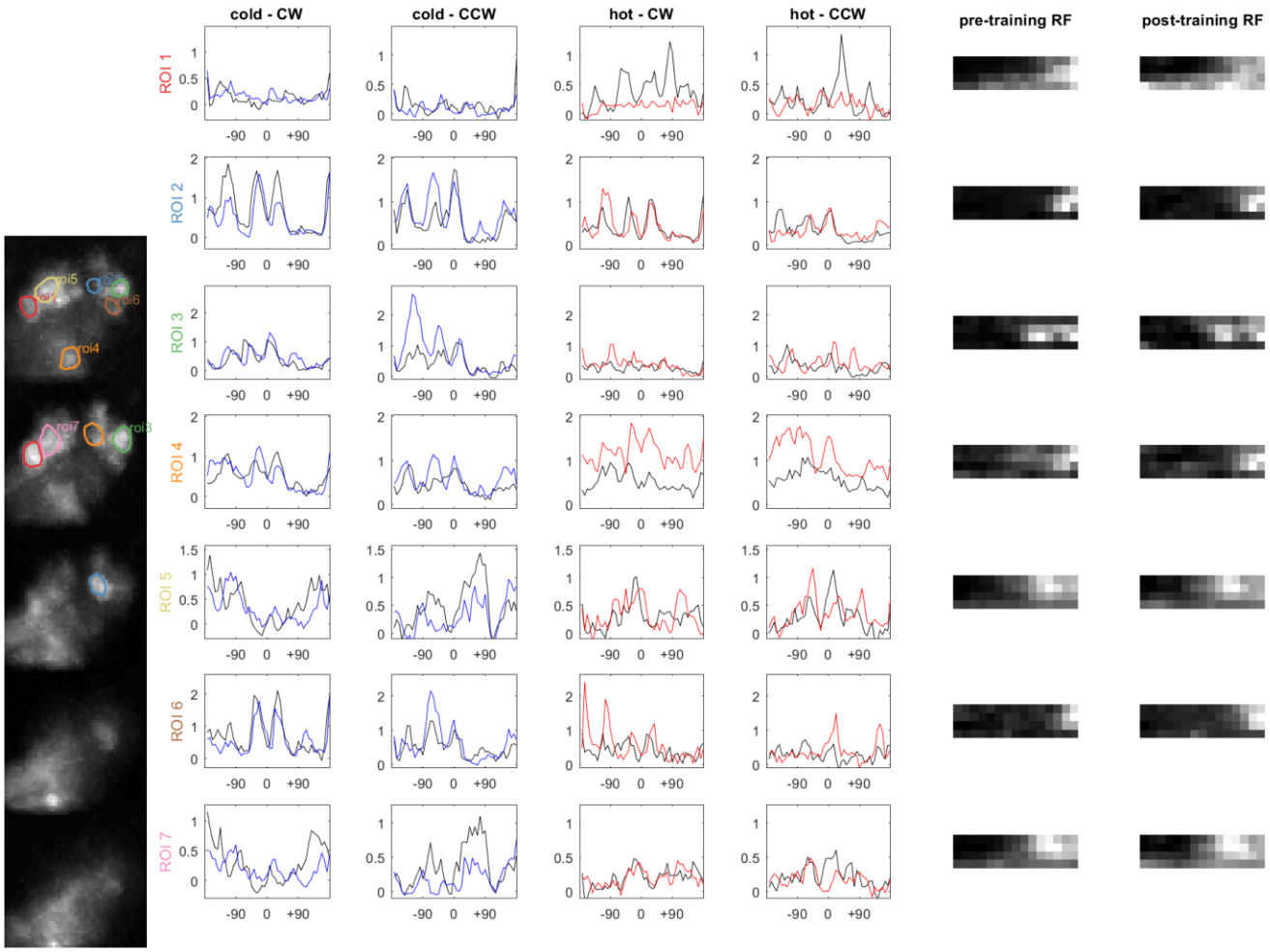
Control Fly 7 – 1 of 2



Control Fly 7 – 2 of 2



Control Fly 8



Control Fly 9